Transcriptional regulation of MRF4 gene expression during embryonic mouse development

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Table of Contents

Summary

Skeletal muscle development in the vertebrate embryo critically depends on myogenic regulatory factors including the bHLH transcription factors MRF4 and Myf5. Both genes exhibit distinct expression patterns during mouse embryogenesis, although they are genetically closely linked with multiple regulatory elements dispersed throughout the common gene locus. MRF4 has a biphasic expression profile. Initially it is expressed in somites and later in fetal skeletal muscles. Transgenic analysis performed in this study demonstrates that elements within a 7.5-kb promoter fragment of the MRF4 gene are sufficient to drive the embryonic wave of expression very similar to the endogenous gene in somites of mouse embryos. In contrast, a 3-kb fragment of the proximal promoter fails to support expression in the myotome suggesting that essential *cis*-acting elements are located between –7.5 and –3 kb upstream of MRF4. Further analysis of this sequence delimits an essential region between –6.6 and –5.6 kb that together with the 3-kb promoter fragment directs transgene expression in the epaxial myotome of all somites during the appropriate developmental period. Deletion analysis of the sequence downstream of the MRF4 promoter allowed to map a 2.8-kb region containing elements crucial for gene expression in the rostral cervical somites. These data provide evidence that the partly overlapping expression patterns of MRF4 and Myf5 in somites are controlled by distinct regulatory elements.

The second phase of MRF4 expression in fetal muscles is controlled by mechanisms that are distinct from those directing the embryonic phase. In the present study all elements necessary for fetal expression map in the region between –11.4 kb and +15.3 kb. Deletion of the +3.9/+15.3 kb sequence results in variable transgene expression in fetal muscles among different transgenic mouse lines. This suggests that elements for stable fetal MRF4 expression involve the region between +3.9 kb and +15.3 kb downstream of the gene.

It is also shown that 11.4 kb sequence upstream of MRF4, including the promoter and the somitic control region identified in this study, is not sufficient to prevent MRF4 activation by the strong distal Myf5 (-58/-48 kb) enhancer.

Hence, additional yet unidentified elements are necessary to convey promoter selectivity and shield the MRF4 promoter from influence by this enhancer.

The role of the Myf5 protein in transcriptional regulation of the MRF4 gene has been studied in mice carrying a Myf5 null mutant allele. Analysis of the Myf5 mutant embryos reveals the role of Myf5 protein for the expression of myotomal markers in the central myotome. Additionally, it is found that Myf5 is required for MRF4 expression in the most rostral cervical somites. In all other somites MRF4 activation does not require Myf5 or MyoD.

1. Introduction

Skeletal muscle development is a well-coordinated process that requires the precise orchestration of myogenic regulatory factors (MRF). Establishing the correct temporal and spatial patterns of each MRF is crucial to myogenesis. Therefore, studying the molecular mechanisms that control the specific expression of MRF genes is essential for understanding how skeletal muscles are formed during embryogenesis.

1.1. Myogenesis in vertebrates

Mammalian skeletal myogenesis proceeds through three main stages: determination or commitment of precursor cells to the muscle lineage, proliferation of myoblasts, and differentiation to myotubes. In vertebrates muscles originate from somites, paraxial head mesoderm, and prechordal mesoderm. Somites are the source for all body muscles, including dorsal, epaxial, deep back and intercostal muscles, hypaxial muscles of the ventral body wall, muscles of the fore and hind limbs, and some of the head muscles. The majority of head muscles are derived from paraxial head mesoderm and prechordal mesoderm (Christ & Ordahl, 1995). Somites arise from presomitic mesoderm and form in pairs as epithelial condensations on either side of the neural tube. In the mouse, the first somite pair forms between E7.5 and E8.0. Within several hours after epithelialization, cells of the ventral part of somites undergo an epithelial-mesenchymal transformation to produce the sclerotome. The sclerotome gives rise to vertebrae and ribs. The dorsal epithelial part of the somite, referred to as dermomyotome, contributes to skeletal muscles, distal ribs and dorsal dermis. Cells in the medial and the lateral parts of the dermomyotome form epaxial and hypaxial somitic buds, respectively. Myotomal progenitor cells leave the somitic buds, form epaxial and hypaxial myotomes, and merge to establish the continuous myotomal sheet (Tajbakhsh & Buckingham, 2000).

Muscle precursor cells are initially maintained in a proliferating, undifferentiated state. Myoblasts of the epaxial myotome form the musculature of the back and do not migrate significantly. On the contrary, some hypaxial myoblasts migrate over relatively long distance from the dermomyotome to their

target areas in the limbs, tongue and the diaphragm where they form muscles. The muscle progenitor cells start to differentiate into myocytes only when they have reached their final destination. Then the cells align, stop dividing, and fuse to form multinucleated myotubes that mature to functional muscle fibers and perform muscle contractions. Primary fibers are formed from embryonic myoblasts at about E13.0. Their role in muscle formation is to serve as a scaffold for secondary fibers that arise at about E16.0. Secondary fibers have their origin in fetal myoblasts. Secondary fibers are associated with primary fibers and share with them a basal lamina. They provide the muscle mass (Patel et al., 2002). A small number of cells, known as satellite cells, are preserved in undifferentiated state throughout life of the organism. The satellite cells are located adjacent to mature myotubes and become activate upon injury or denervation of muscles. Activated satellite cells undergo proliferation and then differentiate to fuse with or replace injured myotubes (Seale and Rudnicki, 2000).

1.2. The myogenic regulatory factors (MRFs)

1.2.1. Molecular structure of MRFs

Specific extrinsic signals from the microenvironment trigger the expression of numerous intracellular factors in precursors that can induce and maintain the myogenic program. The key role in governing myogenesis on the transcriptional level is played by the myogenic regulatory factors (MRFs). This family of transcription factors includes Myf5 (Braun et al., 1989), myogenin (Edmondson & Olson, 1989; Wright et al., 1989), MRF4, which is also described as Myf6 or herculin (Braun et al., 1990a; Miner & Wold, 1990; Rhodes & Konieczny, 1989), and MyoD (Davis et al., 1987). MRFs are basic helix-loop-helix (bHLH) proteins containing two highly conserved and functionally distinct domains, which together make up a region of approximately 60 amino-acid residues. The basic domain mediates binding to *cis*-acting DNA control elements, so called E-boxes (CANNTG), in the promoters or enhancers of target genes. Different families of bHLH proteins recognize different types of E-boxes. Thus experiments in vitro have demonstrated that MRF family members preferably bind to the CAGCTG sequence (Blackwell & Weintraub, 1990). The HLH domain of MRFs mediates dimerization with ubiquitously expressed proteins of

the E2A family. Nonconserved transactivation domains flanking this bHLH region are essential for transcriptional activation of target genes (Braun et al., 1990b; Weintraub et al., 1991; Puri & Sartorelli, 2000; Jones, 2004).

Sequence comparison of MRF family members suggests that the vertebrate genes Myf5, MyoD, myogenin and MRF4 were derived from a single ancestral gene by two gene duplications. Presumably, Myf5 and MRF4 arose from the ancestral MRF gene by gene duplication at the same locus; MyoD and myogenin arose from Myf5 and MRF4, respectively, after a gene duplication event to a second chromosome, and finally, myogenin and MyoD were separated. Therefore, based on sequencing homology, the MRF family can be subdivided in two subgroups, predicting some functional redundancy between Myf5 and MyoD and between myogenin and MRF4 (Atchley et al., 1994).

Each of the MRFs is capable of dominantly inducing myogenesis, when introduced into a variety of non-muscle cells in culture. Overexpression of MRFs leads to the activation of many muscle-specific genes including desmin, M-creatine kinase, troponin I, α-actin, and the acetylcholine receptor subunit genes (Weintraub, 1993). Nevertheless, each of the MRFs plays different, though partially overlapping, roles in vitro and in vivo. Expression analyses in vitro have shown that Myf5 and MyoD are expressed in muscle precursor cells, whereas MRF4 and myogenin are activated only when differentiation of the myoblasts to myocytes is already underway (Weintraub, 1993). This led to the hypothesis that Myf5 and MyoD are required for the determination of myogenic precursor cells, whereas myogenin and MRF4 are responsible for terminal differentiation of myoblasts. Confirming this view, biochemical studies demonstrated that Myf5 and MyoD are capable of remodeling chromatin of muscle-specific genes to allow their transcriptional activation (Gerber et al., 1997). A structurally conserved carboxyl-terminal alpha-helical motif of the differentiation gene myogenin has activity as a general transcriptional activation domain but cannot facilitate the initiation of skeletal muscle gene expression. The helix III of MRF4, which is also considered a differentiation gene, can, however, functionally substitute for helix III of MyoD (Bergstrom & Tapscott, 2001). These data indicate that the MRF4 and MyoD proteins may have an overlapping biochemical function in regulation of myogenesis.

1.2.2. Spatiotemporal expression of the MRF genes during embryogenesis and in adult mice

Expression analysis during mouse embryogenesis and in adult muscle revealed distinct as well as overlapping spatiotemporal expression patterns for MRF genes in muscle cells and their progenitors in somites, visceral arches and limb buds. In mice, Myf5 is the earliest MRF expressed in the somites and limb buds. Its expression starts at E8.0 in the dorso-medial region of the epithelial somites and later spreads throughout the entire myotome and appears in myoblasts of the limbs and branchial arches. Myf5 expression is maintained in the dermomyotome until E11.5 and then rapidly decreases in a craniocaudal direction (Ott et al., 1991; Tajbakhsh et al., 1996a). In adult muscle, Myf5 is expressed in satellite cells and in muscle spindles – stretch-sensitive mechanoreceptors, but not in myonuclei of myofibers. Muscle denervation induces extensive reactivation of the Myf5 gene in myonuclei (Zammit et al., 2004).

Myf5 expression in the myotome is followed by myogenin, which appears in the most rostral, i.e. the most mature somites at E8.5 (Sassoon et al., 1989). Its expression continues at a relatively high level until birth and declines thereafter.

The earliest MRF4 expression is detected during somitic stages 16-19 (ss16-19), trailing Myf5 by 18-24 hours in development. The MRF4 expressing cells are initially localized in the dorsal half of the somite but not within or adjacent to the dermomyotomal lip. The expression then progresses caudally. By ss22-25 a new ventral domain appears in the somitic bud of thoracic somites. Expression in this domain precedes the expression in the dorsal domain of the same somite. Furthermore, in this location MRF4 appears before or simultaneously with Myf5. By ss29-33 in the older thoracic somites MRF4 is expressed through out the whole dorso-ventral extension of the myotome. In younger somites the epaxial expression precedes the ventral expression (Summerbell et al., 2002). After two days MRF4 expression ceases in a craniocaudal direction. In contrast to other MRFs, MRF4 transcripts are undetectable in branchial arches, and in limb buds until E15.5. A second wave of MRF4 expression begins at E14.5 in the neck and anterior back muscles. After E16.5, MRF4 expression strongly increases in all skeletal muscles and MRF4

becomes the predominant MRF expressed in late fetal and adult muscles of the mouse (Bober et al., 1991).

MyoD is the last member of the MRF family expressed in the mouse myotome. Its initial expression is detected in the ventral domain of interlimb somites at E9.5-9.75, where it is initiated in a craniocaudal fashion, with slightly later appearance of transcripts in the epaxial part of occipito-cervical somites (Chen et al., 2001). Then MyoD is expressed at high level until birth in all regions where myogenesis takes place.

The distinct, though partially overlapping expression patterns of four MRFs suggest specific developmental functions for each of these proteins in muscle formation. Therefore, all MRF genes were investigated by targeted mutation in mice.

1.2.3. Targeted inactivation of MRF genes reveals their specific roles in mouse myogenesis

Initially, independent Myf5 mutant alleles were generated in different laboratories (Braun et al., 1992; Tajbakhsh et al., 1996b; Tallquist et al., 2000). All of them demonstrated the delay of myotome formation and early myotomal marker expression until MyoD is activated. Therefore, MyoD was suggested to play a compensatory role, as no major defect in skeletal muscle formation was observed later during development. One of the striking features of the original Myf5 mutants has been the lack of distal ribs resulting in respiratory failure of newborn animals. A more recently generated mutant allele from which the PGK-neomycin selection cassette has been removed by Cre-recombinase resulted in viable and healthy mice, which lacked the rib defect (Kaul et al., 2000). Therefore, it has been concluded that the rib phenotype in the earlier Myf5 mutants was possibly caused by long-distance *cis*-effects of the selection cassette on a yet unknown gene. Development of the myotome was delayed in these mutants. Taken together, analysis of the different Myf5 mutations led to the conclusion that, although Myf5 is essential for the early onset of myogenesis, it is dispensable for the skeletal muscle development because MyoD can substitute for it (Kaul et al., 2000).

MyoD mutant mice do not show any obvious muscle defects at birth, similar to Myf5 mutant mice (Rudnicki et al., 1992). As MyoD mutant embryos

exhibited delayed development of limb musculature, it was proposed that MyoD plays a unique role in the development of muscles arising from migratory precursor cells (Kablar et al., 1997). Adult MyoD mutants have a defect in muscle regeneration through impaired satellite cell function (Megeney et al., 1996; Cornelison et al., 2000).

The essential role of both, Myf5 and MyoD for myogenesis was shown in Myf5:MyoD double mutants. Double homozygous animals developed no skeletal muscle at all, not even mononucleated muscle precursor cells were detected (Rudnicki et al., 1993) demonstrating that either Myf5 or MyoD is required for the determination of mesenchymal cells to the myogenic lineage.

Myogenin deficient mice are immobile and die perinatally because of complete lack of differentiated muscles. Large numbers of mononucleated cells are present in all regions that are normally populated by multinucleated myotubes (Hasty et al., 1993; Nabeshima et al., 1993). In contrast to mice lacking MyoD and Myf5, the early steps of myogenesis occur normally in myogenin deficient mice. Cells can enter the myogenic lineage but are unable to differentiate. Only residual primary myofibers form in myogenin mutants. However, the formation of secondary myofibers, does not take place in homozygous mutant mice (Venuti et al., 1995). Double myogenin/Myf5 and myogenin/MyoD mutants indicate separate functions for these genes and put myogenin genetically downstream of Myf5 and MyoD (Rawls et al., 1995). However, if myogenin is placed under the transcriptional control of the Myf5 promoter, replacing the Myf5 coding sequence ($Myf5^{myog/myog}$), the delay in myotome formation and the rib phenotype of Myf5 mutants is essentially rescued by the myogenin expression under Myf5 specific gene control (Wang et al., 1996). This suggests that myogenin can substitute for Myf5 during early myogenesis and, therefore, appears to have similar biochemical activities as Myf5. On the other hand, double mutants MyoD/$Myf5^{myog/myog}$ are not viable, indicating that myogenin is not fully functionally equivalent to Myf5 (Wang & Jaenisch, 1997). Furthermore, myogenin/$Myf5^{myog/myog}$ double mutants reveal that myogenin expressed under the Myf5 promoter cannot substitute for the lack of endogenous myogenin (Wang & Jaenisch, 1997). This effect may be explained by the downregulation of the Myf5 locus during normal development, leading to reduced myogenin level of the knock-in allele at stages when high myogenin expression is required for

terminal differentiation of myocytes. Hence, this demonstrates the importance of correct spatiotemporal regulation of gene expression during development.

The role of MRF4 in myogenesis has not been clarified by gene disruption analysis. Three different mutant alleles are available and display subtle defects in myogenesis with a slight reduction of the expression of muscle specific genes but no serious muscle problem (Braun & Arnold, 1995; Patapoutian et al., 1995; Zhang et al., 1995). The three alleles were similar in design but surprisingly different in the intensity of their phenotypes ranging from viability of homozygous mice to embryonic lethality. The mildest allele expresses an elevated level of myogenin (Zhang et al., 1995). This observation led the authors to conclude that MRF4 is required for the downregulation of myogenin. Indeed, MRF4 can play a negative regulatory role, as it has been demonstrated in vitro for the cardiac α-actin promoter (Moss et al., 1996). On the other hand, MRF4 expressed under the myogenin promoter is able to substitute for myogenin in activating MRF4 expression and promote formation of myofibers during early myogenesis (Zhu & Miller, 1997). MRF4 is also upregulated in residual muscle fibers that form in myogenin-deficient mutant mice (Rawls et al., 1995). Taken together these observations indicate that these two regulatory factors can mutually compensate for each other and downregulate each other's expression. Whereas mice lacking either MRF4 or MyoD are viable and do not show significant defects in muscle development, MRF4/MyoD double mutants display a severe muscle deficiency similar to that in myogenin mutants (Rawls et al., 1998). This suggests that MRF4 shares a specific myogenic function with MyoD in muscle differentiation. Myogenin or Myf5 cannot compensate for this function. The homozygous myogenin/MRF4/MyoD triple mutants exhibited a more pronounced defect in muscle differentiation than myogenin$^{-/-}$ or MRF4$^{-/-}$/MyoD$^{-/-}$ mice. Myoblasts from these triple mutant animals were unable to differentiate in vitro (Valdez et al., 2000). These observations demonstrate an overlap in functions of myogenin, MyoD and MRF4.

The second MRF4 mutant allele results in a transient deficit in myotome development, which coincides with the first wave of MRF4 expression in somites beginning around E9.0 and ending around E11.0 (Patapoutian et al., 1995). During this period the expression of Myf5, myogenin and MyoD is significantly

reduced in the mutants. Prior to this period and following the time of somitic MRF4 expression, myogenesis appears essentially unaltered. That led the authors to suggest a triphasic model of myotome development: Myf5 initiates the first phase, the second is MRF4 depended, and the third is mediated by MyoD.

All three MRF4 mutant alleles affected Myf5 expression by a *cis*-acting mechanism (Floss et al., 1996; Yoon et al., 1997). The most severe MRF4 mutant allele was a complete phenocopy of Myf5 knockout mice with extensive rib truncations (Braun & Arnold, 1995). Myf5 is localized 8.8 kb downstream of MRF4 on the same mouse chromosome. Obviously, the disruption of chromatin conformation by the recombination events affected Myf5 transcription in MRF4 mutants to varying extent. Moreover, the neomycin cassette inserted in the MRF4 locus might have an effect on a yet unknown gene, as it was described for Myf5 mutations. Indeed, all described MRF4 mutant alleles similarly to Myf5 mutants demonstrated various rib malformations, although neither MRF4 nor Myf5 is expressed in the sclerotome.

Even though data obtained with different MRF4 mutant alleles could not unequivocally determine the exact function of MRF4 in the myotome, they indicated a complex and complicated regulation within the MRF4/Myf5 locus. Therefore one of the key issues is to understand the control elements for both genes and assemble all the sequences in the locus that regulate Myf5 or MRF4 specifically or by a common mechanism.

1.2.4. Transcriptional regulation of MRF genes

A great deal is known about the protein function of the MRFs, but *cis*- and *trans*- regulatory elements that control their expression in the myotome as well as in differentiated muscles are not yet well defined. The regulation of MyoD and myogenin seems to be relatively simple, whereas the MRF4/Myf5 locus control exhibits high complexity.

In the case of myogenin, all of the *cis*-acting sequences required for correct spatial and temporal expression in the embryo lie within 133 bp upstream of the transcriptional start site, although there are additional elements within 1 kb of the promoter that increase the level of expression (Cheng et al., 1993; Yee & Rigby, 1993). E-boxes and MEF2-binding sites within this sequence are crucial

for myogenin activation both in vitro and in vivo (Buchberger et al., 1994; Cheng et al., 1993).

For the MyoD gene, two enhancers were identified. A 258-bp core element that lies 20 kb upstream of the transcriptional start site appears to recapitulate the full expression pattern (Goldhamer et al., 1992, 1995), while a proximal enhancer approximately 5 kb upstream of the promoter drives expression in the medial region of the myotome, and gives delayed expression in limb buds and branchial arches (Tapscott et al., 1992; Asakura et al., 1995). There are three E-boxes within the distal enhancer. Mutation of these E-boxes, however, does not affect the expression pattern of 258-bp/LacZ transgene, suggesting that MyoD activation in the embryo is E-box-independent (Goldhamer et al., 1995). In fact, the 258-bp fragment activates the expression in newly formed somites and limb buds in compound mutant embryos lacking both Myf5 and MyoD (Kablar et al., 1999). Targeted deletion of the mouse core enhancer demonstrated that the enhancer is not required for MyoD expression in nascent myocytes in the epaxial and hypaxial myotomes (Chen & Goldhamer, 2004). Since the core enhancer is the only known MyoD enhancer responsible for the expression in these domains of somites, this observation raises the possibility that additional, yet unidentified regulatory elements exist.

Regulation of the Myf5 gene is extremely complex. The regulatory elements controlling different spatiotemporal aspects of Myf5 expression are dispersed over 200 kb on mouse chromosome 10 (Carvajal et al., 2001; Hadchouel et al., 2000; Summerbell et al., 2000; Zweigerdt et al., 1997). Intensive investigations performed recently allowed to map several regulatory regions in this locus. However, only a few enhancers were located relatively precisely. Three distinct enhancers have been found proximal to the Myf5 promoter (Summerbell et al., 2000). The first of them located within the gene coding sequence of Myf5 functions in the hypaxial domain of somites but drives reporter gene expression incorrectly in the dermomyotome and in the posterior half of the somites. The second enhancer situated between the MRF4 and Myf5 genes initiates transcription in muscle progenitors of branchial arches which subsequently give rise to facial muscles. A third element known as the early epaxial enhancer (EEE) is necessary and sufficient for the initial phase of Myf5 transcription (Teboul et al., 2002, 2003). Another important regulatory region

was mapped between –58 and –48 kb upstream of Myf5 (Hadchouel et al., 2000). This region contains a 270 bp core enhancer that is necessary and sufficient to recapitulate the endogenous expression pattern in limbs and to maintain the expression in somites. The region contains also the element, which is essential to direct the expression in cervical somites and to restrict the transcription appropriately to the myotome (Buchberger et al., 2003). The sequence between –96 and –63 kb is necessary for later expression of Myf5 in head muscles and in a subset of hypaxially derived trunk muscles (Hadchouel et al., 2000). The elements required for Myf5 expression in the ventral domain of the tail somites and the most ventral component of thoracic somites are situated in the region –140 to –88.2 kb (Carvajal et al., 2001). Additionally, it was shown recently that multiple elements are required to regulate Myf5 in adult skeletal muscle. These separate elements are active in different cell populations, similar to the developmental control of Myf5 expression (Zammit et al., 2004). Thus, the sequences responsible for the control of Myf5 expression in muscle spindles were mapped to the –59 to –8.8 kb interval. Other elements, located between –140 kb and –88 kb, are responsible for driving expression in satellite cell-derived myoblasts.

Comparably little is known about the regulation of MRF4 expression. The regulation of this gene appears also rather complex. The proximal mouse MRF4 promoter sequence has been localized within 300 bp upstream of the MRF4 transcriptional start. This region is sufficient to mediate muscle-specific gene activation in vitro (Black et al., 1995). Myogenin, MyoD and Myf5 strongly *trans*-activate this MRF4 promoter. The activation appears to be direct and requires at least one E-box. A similar region (360 bp) was characterized in the rat MRF4 gene. This sequence is also sufficient to induce MRF4 expression in vitro (Naidu et al., 1995). However, in vivo this proximal rat promoter only drives expression in a very limited number (less then 1%) of fetal and adult muscle fibers and is absolutely silent in the myotome (Pin et al., 1997). A 6.5 kb sequence immediately upstream of the mouse MRF4 gene was shown to be sufficient to mediate the second wave of expression in most of fetal trunk muscle but failed to promote early transcription in somites (Patapoutian et al., 1993).

The 8.5 kb proximal promoter sequence of the rat MRF4 gene drives the expression in a subset of myotomal cells of thoracic somites (Pin et al., 1997).

The same sequence also drives the expression in all skeletal muscle. However, fetal myoblast culture reveals a population of transgene-negative myotubes (Pin et al., 1997). The rat sequence between –5 and –4 kb exhibited enhancer activity in cultured myoblasts (Kerkvliet & Hinterberger, 1997). This enhancer drives fast fiber-specific expression in fetal muscle (Pin & Konieczny, 2002).

Evidently, additional regulatory elements are required for the gene expression in all somites. Transgenic analysis of BAC deletion series revealed that the –132/-80 kb region is necessary for the early ventral domain and hypaxial myotomal MRF4 expression. All other aspects of myotomal expression in rostral, thoracic and caudal somites are covered by sequence between –50 kb and 48 kb downstream of the MRF4 gene (Carvajal et al., 2001). All regulatory elements of the MRF4 gene known to date are schematically presented in the Fig. 1.

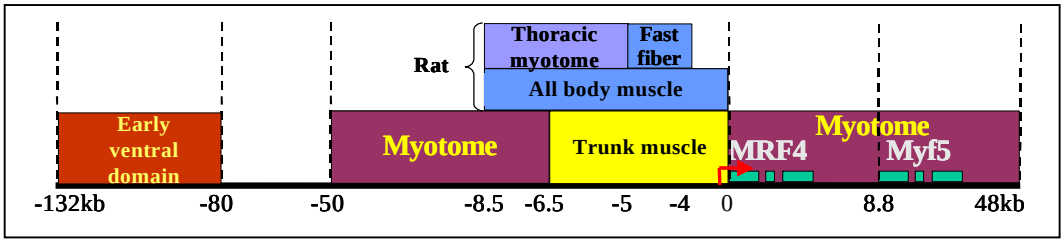

Figure 1. Summary of previously localized regulatory elements of the MRF4 gene. "0" represents the transcriptional start of MRF4. Lower rectangles show the data obtained with the mouse sequence, while three upper rectangles are for the rat sequence. Light green boxes denote MRF4 and Myf5 genes. Red arrow represents the proximal MRF4 promoter (-300 bp). The details and references see in text. The map is not drawn to scale.

Multiple regulatory sequences are present and interspersed throughout the MRF4/Myf5 locus. Some of these regulatory elements could be common for both genes, while others interact specifically with one or the other promoter. For example, elements regulating the expression of Myf5 in branchial arch and limb buds do not activate the MRF4 promoter. This raises the question of how the distal control elements can distinguish between the two promoters to give distinct expression patterns of both genes.

1.3. Aims of the project

The main goal of this project was to localize the elements responsible for the different aspects of the MRF4 gene expression. Previous investigations had demonstrated that regulation of the MRF4 gene is complex and provided approximate mapping of MRF4 regulatory regions (Fig. 1). The ultimate goal of this study was the mapping and detailed characterization of elements regulating MRF4 expression in certain domains of the myotome as well as in fetal and adult muscles.

Another important problem raised in this study was how the selectivity between distal elements controlling the Myf 5 and the MRF4 gene expression and promoters of these genes is established. In vivo the MRF4 gene is not responsive to the upstream Myf5 limb enhancer. This study aimed to understand whether properties of the individual promoters prevent the influence of the enhancer or whether additional shielding elements are required in the locus.

Additionally, this study aimed to elucidate a role of other MRF proteins in the regulation of the MRF4 gene transcription. The gene targeting experiments pointed out that Myf5 and MyoD may be possible candidates to activate MRF4 gene transcription. In this study the Myf5 mutant allele which does not affect rib development (Kaul et al., 2000) was used to monitor development of the myotome, expression of the endogenous MRF4 gene and expression of MRF4 transgenes in the absence of Myf5 protein. Moreover, the potential role of MyoD to compensate for the lack of Myf5 in myogenesis was considered.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

All chemical reagents used in this study were supplied by Merck, Roth, Sigma, Promega and Roche companies, if not mentioned specifically. The most important will be listed below according to subject.

2.1.2. Enzymes

DNAse	M-6101	Promega
RNAse A	7156.1	Roth
Proteinase K	7528.2	Roth

2.1.2.1. Modification enzymes

DNA Polymerase I large (Klenow) fragment	M-2201	Promega
Pfu DNA polymerase	M-7741	Promega
Taq DNA polymerase	M-1861	Promega

T3 RNA Polymerase	P-2083	Promega
T7 RNA Polymerasa	P-2075	Promega

T4 DNA Ligase	M-1801	Promega

2.1.2.2. Restriction enzymes

BamHI	RO-136	New England Biolabs
BglII	RO-144	New England Biolabs
BsrDI	RO-574	New England Biolabs
DraIII	RO-510	New England Biolabs
EcoRI	RO-101	New England Biolabs
EcoRV	RO-195	New England Biolabs
HindIII	RO-104	New England Biolabs
KpnI	RO-142	New England Biolabs

MluI	RO-198	New England Biolabs
NcoI	RO-193	New England Biolabs
NotI	RO-189	New England Biolabs
PmeI	RO-560	New England Biolabs
SfiI	RO-123	New England Biolabs
SpeI	RO-133	New England Biolabs
XhoI	RO-146	New England Biolabs

2.1.3. Vectors

Myf6pClasper	Kindly provided by Dr. R.	(Zweigerdt, 1998)
-20.1/+15.3 kb MRF4 with LacZ	Zweigerdt, TU Braunschweig	
M#6pClasper	Kindly provided by Dr. R.	(Zweigerdt, 1998)
-29/+6.5 kb Myf5 with LacZ	Zweigerdt, TU Braunschweig	
PPD46.21	Kindly provided by Dr. M. Shani,	(Fire et al., 1990)
LacZ gene	Bet Dagan, Israel	
Mouse Myf6 cDNA	Kindly provided by Dr. E. Bober	E. Bober TU-BS
Mouse MyoD pV2C11α	Kindly provided by Dr. T. Braun,	T. Braun
	TU Braunschweig	
PGEM- T Easy Vector System I	A1360	Promega
PGEM- T Vector System I	A3600	Promega
pBluescriptKS+	X52327	Stratagene

2.1.4. Molecular biology reagents

Agarose	2267.4	Roth
Ampicillin	K 029.2	Roth
Labeling Prime-ITII Kit	300385	Stratagene
Fujifilme X-Ray Medical		Fuji
QIAquick PCR Purification Kit	28104	Qiagen
QIAquick Gel Extraction Kit	28704	Qiagen
QIAprep Spin Miniprep Kit	27106	Qiagen
QIAquick Nucleotide Removal Kit	28304	Qiagen
Herring sperm DNA (ssDNA)	D-1811	Promega
Hybond-XL a nylon membrane designed for nucleic acid transfer	RPN2020S	Amersham

2.1.5. In situ hybridization reagents

Anti-Digoxigenin-AP Fab fragments	1093274	Roche
Blocking reagent	1096176	Roche
BM Purple AP substrate	1442074	Roche
Dig-11-UTP	1209256	Roche
Glycine	23390	Serva
Heparin	H-3393	Sigma
Hydrogen peroxide 30%	1.07209.0250	Merck
Levamisol	L-9756	Sigma
Maleic acid	800380	Merk
Methanol	8402	Merck
RNAsin	N-2515	Promega
Sheep serum	S-2263	Sigma
tRNA	109495	Roche

2.1.6. Embryonic cell culture reagents

Albumin bovine serum	A-9647	Sigma
Hyaluronidase	H-3506	Sigma
M2 Medium	M-7167	Sigma
M16 Medium	M-7292	Sigma
Mineral oil	M-8410	Sigma
Penicillin / Streptomycin	P 11-010	PAA
Water for embryo transfer	W-1503	Sigma

2.1.7. Equipment for DNA purification and microinjection

Probe Quant G-50 Micro Columns	27-5335-01	Amersham pharmacia biotech
Ultrafree-MC Centrifugal 0.22μm filter units	UFC30GV25	Millipore

2.1.8. X-gal staining reagents

Deoxycholic acid sodium salt	D-6750	Sigma
Glutaraldehyde 25% aqueous solution	G-6257	Sigma

Igepal CA-630 (NP-40)	I-3021	Sigma
Potassium ferricyanide K3 Fe(CN)6	P-8131	Sigma
Potassium ferricyanide K4 Fe(CN)6	P-9387	Sigma
X-Gal	2315.4	Roth

2.1.9. Equipment and reagents for cryosections

Microscope slides SuperFrost Plus	041300	Menzel-Glaser
Polyfreeze tissue freezing medium	19636	Polysciences Inc.
Cryotome Frigocut	2800E	Jung

2.1.10. Mouse strains

ICR (CD1)	Transgenic animals	Harlan
129 Sv/C57 BL6	Myf5 mutants	(Kaul et al., 2000)

2.1.11. Hormones for superovulation

Intergonan (PMS)	intervet
Pimogonyl-1000 (HCG)	intervet

2.1.12. Anesthesia

Avertin:

Tetraamylalcohol	999	Merk
Tribroniethanol	90710	Fluka

2.1.13. Equipment for microinjections

Borosilicate glass capillaries:

GC 120F-10 1.2mm O.D.x0.69mm I.D.	Harvard apparatus
GC100F-10 1.0mm O.D.x 0.58mm I.D.	Harvard apparatus
Flaming/Brown micropipette puller	Sutter instrument Co.
Transjector	Eppendorf
Cell Tram Air/ Cell tram Oil	Eppendorf

Axiovert 135 microscope	Zeiss
Manipulator	Nicon Narishige
WildM10 stereomicroscope	Leica

2.1.14. Hybridization probes for Southern blot:

Myf5 genotyping	Probe 2 (BamHI/BglII fragment from the MRF4/Myf5 intergenic region)	(Kaul et al., 2000)
LacZ genotyping	KpnI/EcoRV fragment of the LacZ gene	

2.1.15. RNA probes for in situ hybridization.

MRF4	PCR fragment of mouse Myf6 cDNA including 680 bp 3'-end with T3 promoter	antisense T3	E. Bober, TU Braunschweig
MyoD	mouse MyoD pV2C11α cut MluI (749-1785 bp cDNA)	antisense T3	T. Braun, TU Braunschweig
En1	En1 5'probe cut Hind III	antisense T7	K. Schughart, GSF Obershleissheim
Sim1	Sim1 probe cut EcoRV 700 bp	antisense T7	A. Sander

2.1.16. Primers used in this study

Genotyping of transgenes, LacZ:

LacZ 5'-1	5'CGTTACCCAACTTAATCGCC
LacZ 3'-1	5'AGGATGATGCTCGTGACGG

Genotyping of Myf5 mutants:

Pre AflII	5' GCAGCATGGAACTGCTTCC
Post MluI	5'ACTGTACCCCTATTGGGACC
Ex2-3'	5' TTTCGGGACCAGACAGGGC

Myf6 probe template:

Myf6 probe	5' GTGGCCAAGTGTTTCGG
M13reverse	5' GAAACAGCTATGACCATG

Ura3 sequencing:

LacZ end 5’ TATCTTATCATGTCTGGATCG

3’Ura 5’ CAATTCTGCTAACATCAAAAGG

Post KpnI 5’ ACGTGTTCCTCTCCACTGC

MRF4-VII:

NcoI (3908) 5’ CATGGAAAGATCATAGACAAC

BglII (5795)* 5’ **GTTTAAAC**AGATCTCCATAACTGGGACC

MRF4-IX:

NcoI (3908) 5’ CATGGAAAGATCATAGACAAC

BsrDI (4950)* 5’ **GTTTAAAC**TGCATATCCCACTCATTCG

MRF4-X:

DraIII (4783) 5’ ATGAGTGTGATGTGCTGA

BglII (5795)* 5’ **GTTTAAAC**AGATCTCCATAACTGGGACC

*Sequence in bold indicates constant Pme I site for subcloning.

Genotyping for MRF4-XI

PostPmeI 5’ AGAAATCTCTTTATGTCCAGG

PrePmeI 5’ TGTTGAATGTTTAGGAGGCC

Myf6LacZ for MRF4-XIII and MRF4-XIV constructs (without Ura3 gene)

SalI 5’ 5’-AGCTGGGTCGACTTATGTCAC

LacZ-NotI 3’ 5’-AG**GCGGCCGC**TTTGACTTTGTCGATCCAG

Sequence in bold indicates constant Not I site for subcloning.

Myf5 enhancer (Buchberger et al. 2003)

Myf5-IV forward 5’-GCCTGCCTTTAACGCAGTGTGTC

Myf5-IV reverse 5’-GGTAGATAGGAGCCTCAAAATAG

2.1.17. Algorithms and databases used in this study.

webbcutter	http://www.firstmarket.com/cutter/cut2.html	
BLAST	http://www.ncbi.nlm.nih.gov/BLAST/	Altschul et al., 1990
Blast 2 sequence	http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html	Tatusova & Madden, 1999
Blast human genome	http://www.ncbi.nlm.nih.gov/genome/seq/HsBlast.html	
Pubmed	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi	
Gene regulation	http://www.gene-regulation.com/	
transfac	http://www.biobase.de/pages/products/transfac.html	
MatInspector Genomatix	http://www.genomatix.de/cgi-bin/matinspector.html	Quandt et al., 1995
ECR browser	http://ecrbrowser.dcode.org/	Ovcharenko et al., 2004

2.2. Methods

2.2.1. Molecular biology methods

All standard molecular biology techniques used in this study were performed either according to the standard protocols (Sambrook et al., 1989) or according to manufactures instructions. Plasmid DNA was purified using QIAprep Spin Miniprep Kit. DNA fragments were isolated from agarose gel using QIAquick Gel Extraction Kit. PCR products were purified using QIAquick PCR Purification Kit. Subcloning of DNA fragments was performed according to Promega protocols. Filling of 5'-protruding ends with dNTP by Klenow fragment for blunt- end cloning was performed according to Promega protocol. Plasmids were transformed into E. *coli* XL1- Blue line competent cells (Stratagene).

2.2.1.1. Genomic DNA isolation from adult and embryonic tissues

DNA from tissue- piece of tail, embryonic yolk sack or fetal skin was isolated by treatment with 0.4 mg/ml Proteinase K in the lysis buffer and following extraction with phenol: chloroform. DNA was precipitated in isopropanol, washed once with 70% ethanol and dissolved in the 0.1x TE buffer. Further analysis was done using PCR or Southern Blot hybridization.

Lysis buffer:

0.1 M Tris pH 8.0

5 mM EDTA pH 8.0

0.2% SDS

0.2 M NaCl

1x TE buffer:

10 mM Tris pH 8.0

1 mM EDTA pH 8.0

2.2.1.2. Genotyping

Genotyping of transgenic mice and Myf5 mutants was performed by Southern analysis (see sections 2.2.1.2 and 2.1.14) or by PCR (see sections 2.2.1.3 and 2.1.16).

2.2.1.3. Southern blot hybridization

Genomic DNA was digested with EcoRI for genotyping of transgenes or with BamHI for genotyping of Myf5 mutants. The digested DNA was separated electrophoretically on 0.7% agarose gel in the TAE buffer. Following electrophoresis DNA was depurinated in 0.25 M HCl 15 min and denaturated with 0.4 N NaOH 20 min. Transfer DNA to the nylon membrane was performed in 0.4 N NaOH over night following UV crosslinking. Then the membrane was washed 1 hour in the washing buffer at 65°C and 1 hour in the Church buffer at 65°C. DNA probe was labeled with [α- ^{32}P] dCTP (Amersham) using the Strategene random prime labeling kit according to manufacturer recommendation. The labeled probe was purified using QIAquick Nucleotide Removal Kit. Hybridization was performed at 65°C for 16 hours in the Church

buffer. Then the membrane was washed in the washing buffer 4 times for 15 min each at 65°C and exposed to X-ray Fuji film.

Genotyping of transgenes with LacZ probe (KpnI/ EcoRV fragment) detected the 4 kb band. Genotyping of Myf5 mutants with Probe2 as described by Kaul et al. (Kaul et al., 2000) detected two bands: 15 kb wild type and 11 kb mutant.

50xTAE buffer:

242 g Tris

57.1 ml Acetic Acid

0.05 M EDTA pH 8.0

Washing buffer:

0.04 M NaH_2PO_4 pH 7.6

1% SDS

Church buffer:

0.5 M NaH_2PO_4 pH 7.6

1 mM EDTA

7.5% SDS

0.1% ssDNA

2.2.1.4. PCR.

PCR was used to amplify DNA for the cloning purpose, to obtain the template for RNA probe, to check the orientation of the subcloned DNA fragments and to genotype the Myf5 mutatns and transgenic embryos or mice. Proofreading Pfu DNA polymerase was used to generate DNA fragments for subcloning purposes. The resulting PCR products were briefly treated with Taq polymerase and dATP as recommended by the manufacturer (Promega) for further subcloning in TA cloning vector. All other PCR reactions were performed with Taq polymerase.

Example of a standard PCR protocol:

- 3 min 95°C initial denaturation
- Cycling 30 cycles:
- 30 sec 95°C
- 30 sec 55-65°C (annealing temperature depends on the melting temperature of the primers used)

- 1 min extension time at 72°C for each kilobase
- 10 min final extension at 72°C

Genotyping of transgenic embryos and mice was done by PCR with primers LacZ 5'-1 (forward) and LacZ 3'-1 (reverse) that amplified 1 kb sequence of the LacZ gene.

Genotyping of Myf5 mutants was done by PCR with the primers Pre AflII, Post MluI and Ex2-3'. Wild type allele was identified by PCR with the primers Pre AflII (forward) and Post MluI (reverse) resulting in 500 bp PCR product. The mutant allele was identified by PCR with the primers Pre AflII (forward) and Ex2-3' (reverse) resulting in 850 bp PCR product.

2.2.1.5. Construction of MRF4 transgenes

Two YACs of different length were derived from clone ICRFy 903B1022 containing the mouse MRF4/Myf5 gene locus using homologous recombination with B1 repetitive elements in yeast as reported previously (Zweigerdt et al., 1997). Insertion of the nlacZ reporter gene into the AUG start codon of the MRF4 gene was also performed by homologous recombination (Zweigerdt, 1998). Briefly, the MRF4 targeting vector was constructed by fusing the 217-bp SalI/KpnI fragment of MRF4 exon 1 in frame to the nlacZ gene in plasmid pPD-46.21 (Fire et al., 1990). The yeast selection marker Ura3 was isolated as BamHI fragment from plasmid pYDp-U (Berben et al., 1991), linked to the 2.8-kb KpnI/BamHI fragment from the MRF4 gene, and the combined fragment was then cloned downstream of nlacZ in the above vector. For yeast transformation the entire recombination cassette was excised from the vector. Upon selection of recombinant YACs and their characterization by Southern blot analysis, the MRF4-nlacZ reporter gene including approximately 20 kb upstream and 15 kb downstream flanking sequence was recuperated from the recombinant YAC and subcloned into a cosmid vector pClasper by standard recombinant DNA technology (Sambrook, 1989). The resulted vector Myf6 pClasper (Zweigerdt, 1998) was used to obtain individual MRF4 transgenes.

Transgenic reporter fragments were digested away from the pClasper vector backbone with NotI (for construct MRF4-I); with SfiI/NotI (for construct MRF4-II); with XhoI/NotI (for construct MRF4-III); with XhoI/SpeI (for

construct MRF4-IV); with PmeI/SpeI (for construct MRF4-V); with NcoI (for construct MRF4-VI); with BglII (for construct MRF4-VIII) and with BamHI (for construct MRF4-XII) (Fig. 3). The NotI site belongs to the pClasper sequence, one of the NcoI sites is from the Ura3 gene sequence, and all other listed restriction sites are from the MRF4 locus genomic sequence.

For constructs MRF4-VII, MRF4-IX, and MRF4-X subfragments were generated by PCR using proofreading Pfu DNA polymerase (Promega) and the appropriate primers centered around the indicated restriction sites (see Section 2.1.16 and Fig. 3). The PCR products were subcloned into the pGEM-T easy vector. Resulting plasmids were digested with SpeI and PmeI in order to insert the PmeI/SpeI MRF4-LacZ reporter fragment (MRF4-V).

Construct MRF4-XI was obtained from MRF4-VII, which was linearized with Pme I. Then the BglII/PmeI restriction fragment of the locus was introduced (blunt-end ligation) in the PmeI site of the MRF4-VII construct.

For the MRF4-XIII construct the PCR fragment Myf6LacZ including SalI/KpnI part of the MRF4 gene and the LacZ gene was subcloned into the pGEM-T easy vector. The XhoI/SpeI restriction fragment from M#6pClasper was subcloned into pBluescriptKS+. This resulting plasmid was linearized with SalI and NotI and the SalI/NotI fragment from Myf6LacZ was subcloned.

MRF4-XIV was generated from MRF4-XIII. Vector pKS-MRF4-XIII was linearized with ApaI and PmeI. Then the ApaI/PmeI restriction fragment from MRF4-X was subcloned.

The Myf5 enhancer sequence was amplified by PCR and subcloned into the pGEM-T easy vector as described previously (Buchberger et al., 2003). Then the resulting vector was linearized with SpeI and the PmeI/SpeI MRF4-LacZ reporter fragment (MRF4-V) or the XhoI/SpeI MRF4-LacZ reporter fragment (MRF4-IV) constructs were subcloned (blunt-end ligation).

All listed above constructs were digested away from the plasmid backbone with NotI prior to microinjections (for further procedure see Section 2.2.2.3).

2.2.1.6. Whole-mount in situ hybridization

Whole-mount in situ hybridization on mouse embryos was carried out using digoxigenin-labeled riboprobes according to the protocol of Wilkinson (Wilkinson, 1992) with some modifications.

Embryos were harvested at E9.0-E13.0, dissected in ice cold PBS. Yolk sacks were collected for DNA analysis. Embryos were fixed overnight in 4% formaldehyde, pH7.5 at +4°C on shaker. After fixation embryos were washed twice in PBT (PBS + 0.1% Tween) dehydrated gradually in methanol and stored in 100% methanol at -20°C.

Before hybridization embryos were rehydrated gradually and washed twice in PBT, then bleached with 6% H_2O_2 in PBT for 30 min, and then washed again with PBT twice. Proteinase K (PK) treatment was performed with 10 μg/ml PK in PBT at RT for E9.0-9.5 3 min, for E10.5 6 min, for E11.5 10min, for E12.5 12min and for E13.0 15 min. After PK treatment the reaction was stopped with 2 mg/ml Glycine in PBT. Then embryos were washed twice in PBT and fixed in 0.2% gluteraldehyde / 4% formaldehyde in PBT for 20 min. After fixation embryos were washed twice with PBT, then with 1:1 PBT: hybridization buffer at RT and replaced in hybridization buffer at 70°C. After 1 hour embryos were transferred into hybridization buffer containing 1 μg/ml digoxigenin-labelled riboprobe. Hybridization was performed on shaker at 70°C for 16-18 hours.

After the hybridization unbound probe was removed by multiple washes. First two washes in solution 1 at 30 min, then 10 min in 1:1 solution 1: solution 2 at 70°C and three times in solution 2 for 5 min at RT, following treatment with 100 mg/ml RNAse in solution 2 at 37°C for 30 min. Then embryos were washed with solution 2 and solution 3 for 5 min each at RT and then twice in solution 3 at 65°C for 30 min. Following this embryos were washed three times in MABT with 2 mM levamisole, then incubated 3 hours in MABT with 2% blocking solution and 20% goat serum at 4°C and then overnight in similar solution with 1:1000 anti-DIG Fab fragments on shaker.

Unbound antibodies were removed by washing eight times for 1 hour each with large volumes of MABT with 2 mM levamisole. Prior staining embryos were washed three times with NTMT for 10 min each at RT. Embryos were stained in BM purple AP substrate with 2 mM levamisole and 1% Tween at

RT in dark place. Stained embryos were washed with PBS and fixed with 4% formaldehyde.

Solutions used for in situ hybridization:

PBS (1 liter)

7.59 g NaCl

0.99 g Na_2HPO_4

0.4 g NaH_2PO_4

pH 7.5

20xSSC (1 liter)

175.3 g NaCl

88.2 g Na citrate

Hybridization buffer:

50% formamide

5xSSC

1%SDS

50 μg/ml yeast RNA

50 μg/ml heparin

pH 6.0

Solution 1:

50% formamide

5xSSC

1%SDS

Solution 2:

0.5 M NaCl

10 mM Tris pH 7.5

1% Tween

Solution 3:

50% formamide

2xSSC

MABT

100 mM Maleic acid

150 mM NaCl

0.1% Tween 20

Blocking solution, stock:

10% blocking reagent in MAB

NTMT:

100 mM NaCl

100 mM Tris pH 9.5

50 mM $MgCl_2$

1% Tween

2 mM levamisole

2.2.2. Production of transgenic mice

Transgenic mice were generated by pronucleus injection of single- cell embryos as described by Hogan et al. (Hogan et al. 1994) with some modifications.

2.2.2.1. Superovulation of mice

The gonadotropins PMS and hCG were injected intraperitoneally 5 IU of each into CD1 females at 14.00 with 48 hours interval between the PMS injection and hCG injection. After the administration of the hCG, one female was placed in a cage with one male, and female was checked for a copulation plug the next morning.

2.2.2.2. Collecting fertilized eggs

Pregnant female mice were sacrificed humanely on the morning after copulation. Oviducts from several mice were collected into embryological watch glass containing M2 medium. The eggs surrounded by cumulus cells were released by tearing the ampulla of the oviduct with fine forceps. Eggs were treated with 300 μg/ml hyaluronidase in M2 medium to remove the cumulus cells. Then eggs were washed 3 times in M2 medium from the rest of hyaluronidase and transferred into drops of M16 medium covered with mineral oil. Eggs were incubated in M13 medium at 37°C in 5% CO_2 prior and after pronucleus injection.

2.2.2.3. Preparing DNA samples for microinjections

Vector sequence was removed from all constructs prior to injections by digestion with Not1 restriction enzyme or other restriction enzymes (see section 2.2.1.5) and separation of DNA fragments on 0.8% agarose gel. DNA fragments carrying the transgenes were isolated by electroelution in dialysis bags, purified twice with phenol/chloroform and once with chloroform alone. Recovered DNA was dissolved in 10mM Tris-HCl, pH 8.5, run over a ProbeQuant G-50 column (Amersham Biosciences), reprecipitated and washed several times with ethanol. Purified fragments were dissolved in 0.1x TE buffer made with sterile water specified for embryo transfer (Sigma). Prior to microinjections DNA was diluted to the final concentration 0.5-1 ng/μl in 0.1x TE buffer and filtered by Ultrafree-MC Centrifugal 0.22μm filter units.

2.2.2.4. Microinjecting DNA into pronuclei

For injection eggs were transferred in the injection chamber containing the M2 medium covered with mineral oil and placed on the Axiovert 135 stage. Then one egg was immobilized by the holding pipette connected to Cell tram Oil pump. The injection pipette filled with DNA solution was introduced into one of the pronuclei. DNA flow was administered by the Transjector 5246 (Eppendorf). After injections eggs were transferred into the M16 medium and placed into the incubator for recovering.

2.2.2.5. Transfer of embryos into oviduct.

Injected embryos at one-cell stage (the day of injection) or two-cell stage (next day after injection) were transferred into the infundibulum of the oviduct of the pseuodopregnant recipient (foster). For this end, female recipient mice have been mated to vasectomized males the evening before the transfer. Recipient mice were anaesthetized by intraperitoneal injection with 0.017 ml of 2.5% avertin per gram of body weight. Embryos were transferred into the M2 medium and with help of mouth pipette were introduced into the infundibulum of the oviduct. From 10 to 13 embryos were transferred per each oviduct.

Mice were sacrificed at different developmental stages or conducted until birth in order to obtain stable transgenic lines.

2.2.3. Analysis of transgene expression

2.2.3.1. Whole mount staining for β-galactosidase activity

Embryos were collected at various developmental stages counting the day of transfer as E0.5. Isolated embryos at E9.0- E14.0 were fixed in 0.2% glutaraldehyde dissolved in buffer B for 10-30 minutes at room temperature. Fixed embryos were then washed in three changes of buffer C for 30 minutes each, and incubated in staining solution overnight at room temperature. After staining embryos were washed in buffer C.

Embryos E15.5- newborns were fixed in 0.2% glutaraldehyde and 1% formaldehyde dissolved in buffer C overnight, then skinned, dissected and fixed in fresh fixative solution for 1- 2 hours. Then they were treated by same protocol as smaller embryos.

Buffer B:

5 mM EGTA

2 mM $MgCl_2$

in PBS.

Buffer C:

5 mM EGTA

2 mM $MgCl_2$

0.01% sodium desoxycholate

0.02% Nonidet P-40

in PBS.

Staining solution:

5 mM $K_3Fe(CN)_6$

5 mM $K_4Fe(CN)_6$

0.1% X-gal

in buffer C.

2.2.3.2. Vibratome sections of stained embryos

Stained embryos were fixed in 4% formaldehyde in PBS for 1 hour, and then washed 3 times in PBS for 20 min each. Then they were incubated in embedding medium on shaker overnight at +4°C. Next day embryos were

embedded in fresh embedding medium with 2.5% glutaraldehyde for polymerization. Embryos were cut at 40-55 μm using a vibratome Leica VT 1000s.

Embedding medium:

0.5% gelatin
30% chicken albumin
20% sucrose
in PBS.

2.2.3.3. Tissue section staining for β-galactosidase activity

For cryosections different muscles from adult mice were frozen fresh in liquid nitrogen. In some cases tissue were embedded in Polyfreeze tissue freezing medium. Frozen tissues were cut at 12-20 μm using the Jung Frigocut 2800E cryotome. Sections were dried 30 min at room temperature, fixed 10 min in 0.2% glutaraldehyde and 1% formaldehyde dissolved in buffer C, washed in buffer C 3 times for 10 min each and stained in staining solution overnight at room temperature. After staining sections were washed in buffer C, stained briefly with eosin and mounted in gelatin.

2.2.3.4. Photomicroscopy

Pictures of whole-mount embryos were taken under a Leica MZ 12 stereomicroscope using a Polaroid 3CCD color camera and Polaroid DMC2 software. Sections were photographed on a Leica DM-RBE microscope equipped with a digital camera ProgResC12. Figures were assembled with Corel DRAW.

3. Results

3.1. Characterization of control elements responsible for myotomal expression of the MRF4 gene

3.1.1. Endogenous expression of the MRF4 gene

In order to investigate the regulation of the MRF4 expression, it is necessary to know the endogenous expression of this gene. The expression pattern of the MRF4 gene was studied previously using radioactive in situ hybridization on tissue sections and by RT-PCR (Bober et al., 1991; Hinterberger et al., 1991). Detailed whole-mount expression patterns of embryonic stages E9.0-9.5 are described by Rigby and colleagues (Summerbell et al., 2002). Although these data were informative, they do not provide a complete control set for the transgenic experiments presented here, since only the early stages of the expression were described in detail. Thus, to obtain the whole-mount image of the MRF4 expression throughout development of the myotome whole-mount in situ hybridizations were performed. Embryos on E9.5, E10.5, E11.5 and E12.5 were hybridized with a digoxigenin-labeled MRF4 antisense RNA probe, corresponding to a 680 bp 3'-end fragment, the same probe used by Bober et al. (Bober et al., 1991). The results shown in Fig. 2 indicate that the MRF4 expression was restricted to myotomal cells. MRF4 transcripts first appeared in the central region of the myotome in rostral somites, later progressing in rostro-caudal direction. At the same time the expression spread within individual somites in hypaxial and epaxial directions. The early ventral domain in caudal somites described previously (Summerbell et al., 2002) was barely detectable in my experiments. The two most cranial, oldest somites showed a lower level of MRF4 expression than all other somites throughout development. At E10.5 the expression spread across the full dorso-ventral extent of the myotome. Then the expression decreased gradually in cranio-caudal direction. At E12.5 only the youngest somites of mice still expressed MRF4. These data are in agreement with previously published results and provide a complete control set for the transgenic experiments described here.

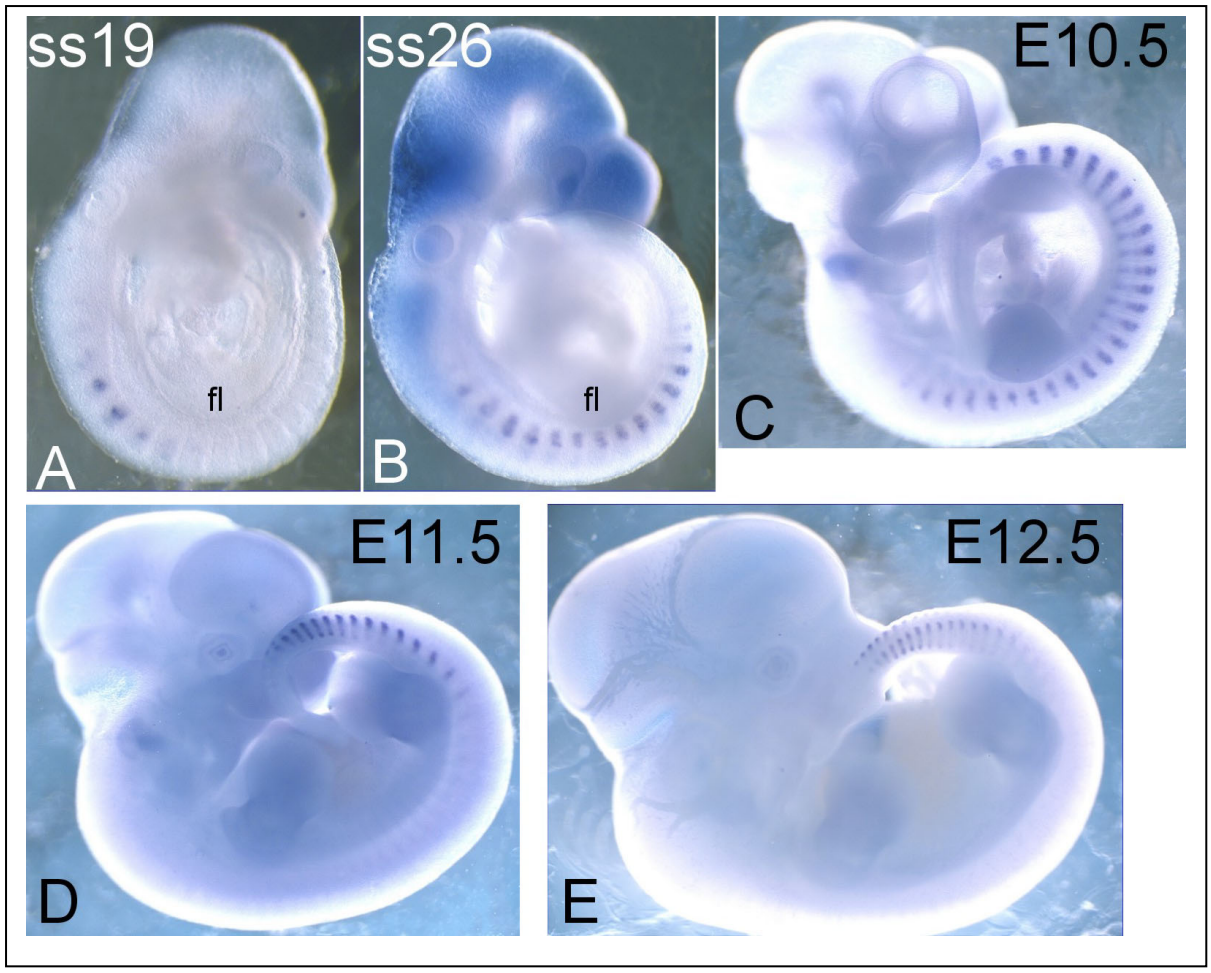

Figure 2. Endogenous expression of the MRF4 gene. Expression is determined by in situ hybridization with digoxigenin- labeled antisense RNA probe (dark blue staining). MRF4 first appears in the central part of the myotome of rostral somites at ss19 (A). At ss26 expression spreads in dorso-ventral direction within each somite and progresses to newly formed somites in caudal direction (B). At E10.5 expression appears in the whole myotome of all somites (C). At E11.5 expression rapidly ceases in rostro-caudal direction (D). At E12.5 only young tail somites still express the MRF4 gene (E). “fl”, forelimb. The apparent staining in the head (B) is an artifact.

3.1.2. Generation of transgenic constructs and transgenic mice

The main goal of this study was to characterize regulatory elements controlling MRF4 expression throughout mouse development. To achieve this transgenic mouse technology was applied. This strategy allows to determine the function of the tissue-specific regulatory elements at different stages of embryonic development and in adult animals. In order to investigate the spatiotemporal regulation of MRF4 expression, chimeric mice carrying the MRF4/Myf5 locus on yeast artificial chromosomes (YACs) of approximately 600 kb length was initially analyzed in our laboratory (Zweigerdt, 1998; Zweigerdt et al., 1997). Two YACs carrying the nLacZ reporter gene inserted into the MRF4 open reading frame (KpnI site) plus approximately 500 kb downstream sequence and 85 kb or 36 kb upstream sequence, respectively, showed comparable myotomal expression in somites of E9.5/E10.5 embryos (Fomin et al., 2004), which appeared very similar to the endogenous MRF4 gene. However, details of the expression patterns could not be assessed due to the chimeric nature of these transgenic embryos. Removal of most sequence downstream of Myf5 had no appreciable effect on MRF4-reporter activity (data not shown) indicating that expression was essentially driven by sequences located within 36 kb 5'-upstream and approximately 15 kb 3'-downstream of the gene. To further delineate important control regions, a series of MRF4 transgenes in plasmids containing promoter fragments and 3' gene flanking sequences of various lengths was constructed in the present study (Fig. 3). The individual constructs exhibited fairly consistent expression patterns in somites of multiple transient transgenic embryos and stable mouse lines (see Table 1), although ectopic expression domains and variations in signal intensity were observed occasionally. The largest construct MRF4-I encompassed 20.1 kb upstream and 15.3 kb downstream sequence of the MRF4 gene. Other constructs were obtained by deletion of certain fragments from MRF4-I with appropriate restriction endonucleases or by subcloning the DNA fragments generated by PCR to the construct containing the so-called minimal promoter (see Section "Material and methods").

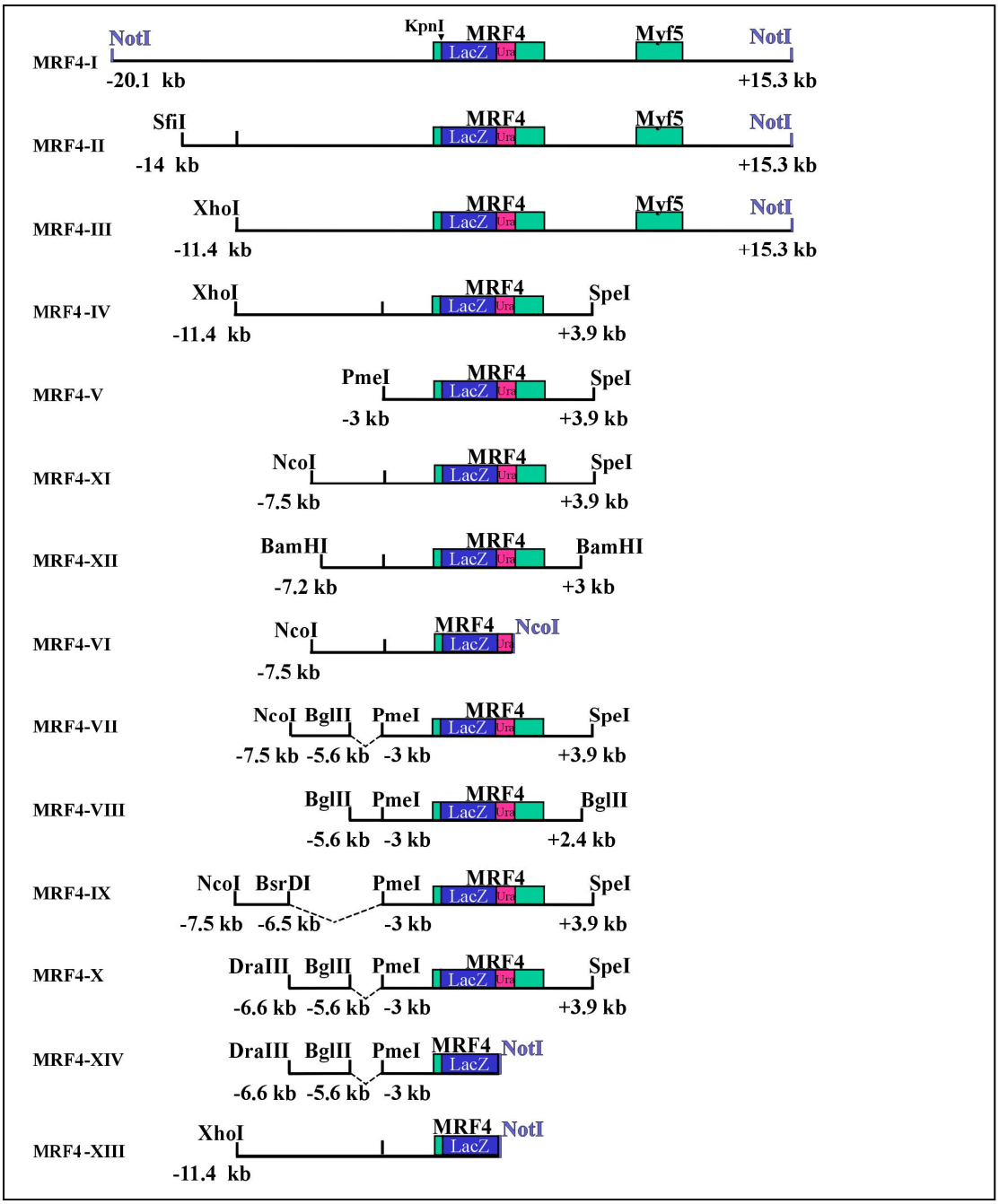

Figure 3. Schematic representation of MRF4 transgenes. A series of plasmid-based transgenes was constructed using a 35.4-kb fragment as starting material that was recuperated from the recombinant YAC encompassing the modified MRF4 gene. This and successively shorter fragments were obtained by digestion with the indicated restriction enzymes. Internal deletions of the promoter fragment (MRF4-VII, MRF4-IX, MRF4-X and MRF4-XIV) were generated by PCR using primers at the indicated restriction sites. Different transgenes are referred to by Roman numerals. Coordinates of the cloned fragments are given relative to the MRF4 gene counting the first nucleotide of the cDNA as +1. Light green boxes mark MRF4 and Myf5 genes, dark blue box marks nLacZ gene and red box marks the Ura3 gene used as a selection marker in the yeast. The restriction sites, NotI and NcoI, denoted by light gray belong to the vector and to the Ura3 gene sequences, respectively. The construct MRF4-XIII was examined only in fetal stages. The maps are not drawn to scale.

Table 1. Summary of transgenic embryos expressing β-galactosidase in somites. Embryos that were positively genotyped for the LacZ transgene and expressed β–galactosidase in somites are listed, except for MRF4-V and MRF4-VIII, which failed to express the reporter but contained the transgene. Numbers in parenthesis refer to total of transient embryos and lines examined at different stages. [a] Variable ectopic expression was observed mostly in head mesenchyme and rarely in neural tube and branchial arches at various developmental stages.

Transgene	Transient	Line	Somitic expression				Ectopic[a]
			E9.5	E10.5	E11.5	E12.5	
MRF4-I	12	1	2(2)	5(5)	6(6)	3(3)	6(13)
MRF4-II	7	1	2(2)	3(3)	3(3)	3(3)	1(8)
MRF4-III	7	1	2(2)	4(4)	3(3)	2(2)	2(8)
MRF4-IV	12	3	3(3)	7(7)	10(10)	4(4)	2(15)
MRF4-V	18	4	0(4)	0(10)	0(10)	0(10)	1(22)
MRF4-VI	16	2	3(3)	8(8)	8(9)	3(4)	5(18)
MRF4-VII	4	3	4(4)	4(4)	5(5)	3(3)	2(7)
MRF4-VIII	8	1	0(5)	0(5)	0(1)	0(1)	1(9)
MRF4-IX	9	4	6(6)	7(7)	6(7)	4(5)	4(13)
MRF4-X	7	2	3(3)	5(5)	4(4)	3(3)	2(9)
MRF4-XI	2	3	3(3)	4(4)	4(4)	3(3)	2(5)
MRF4-XII	-	2	2(2)	2(2)	2(2)	2(2)	1(2)
MRF4-XIV	3	-	1(1)	1(1)	1(1)	-	0(3)

3.1.3. The sequence between –20.1 kb and +15.3 kb of the MRF4 gene is sufficient to recapitulate most of the embryonic expression pattern of the gene

Transgene MRF4-I encompassed 20.1 kb upstream and 15.3 kb downstream sequence of the MRF4 gene. The transgene displayed faithfully the dynamic expression pattern in somites of embryos between E9.0 and E12.5 very similar to the endogenous gene as demonstrated by in situ hybridization (compare Fig. 4 with Fig. 2). This result was obtained in multiple transient embryos and one stable line (see Table 1). MRF4-I expression was first initiated in the embryo at somite stage 19 (E9.0) within the dorsal myotomal half of the most rostral somites (Fig. 4 A) but not in the early epaxial domain (Fig. 4 C) that is characteristic for Myf5. With further development, expression rapidly proceeded into more caudal somites and expanded also dorsally and ventrally within individual somites (Fig. 4 B, D). By E10.5 transgene expression had reached the

ventral myotome in thoracic somites (Fig. 4 E). At E12.5, expression was down-regulated in rostro-caudal direction reflecting the transient wave of embryonic MRF4 expression in somites (Fig. 4 G). Since LacZ protein is presumably more stable than MRF4 mRNA, the expression of the transgene at E11.5 was observed in more somites compared with the results of in situ hybridization (compare Fig. 4 F with Fig. 2 E). It should be emphasized that the early hypaxial domain of the MRF4 expression in thoracic somites of E9.5 embryos, which was previously identified by in situ hybridization (Summerbell et al., 2002) and with a BAC transgene (Carvajal et al., 2001) was not detected with MRF4-I (Fig. 4 B, C). This domain was actually not found in any of the transgenes used in this study. This fact supports the notion that the early ventral expression of MRF4 depends on a far upstream regulatory element located in the –132 to –80 kb sequence interval. However, later at E10.5 and E11.5 expression was observed in the hypaxial domain in most of the transgenic embryos suggesting that the early ventral domain and the hypaxial domain of mature myotomes are regulated in different ways. It is also noteworthy that MRF4-I was not activated in branchial arches and in the epaxial dermomyotomal lip of most transgenic embryos, although the intergenic enhancers driving Myf5 expression in branchial arches and in the early epaxial domain are present in the construct. This suggests that insulation of the MRF4 promoter from the branchial arch and the early epaxial enhancer is maintained in the context of this transgene.

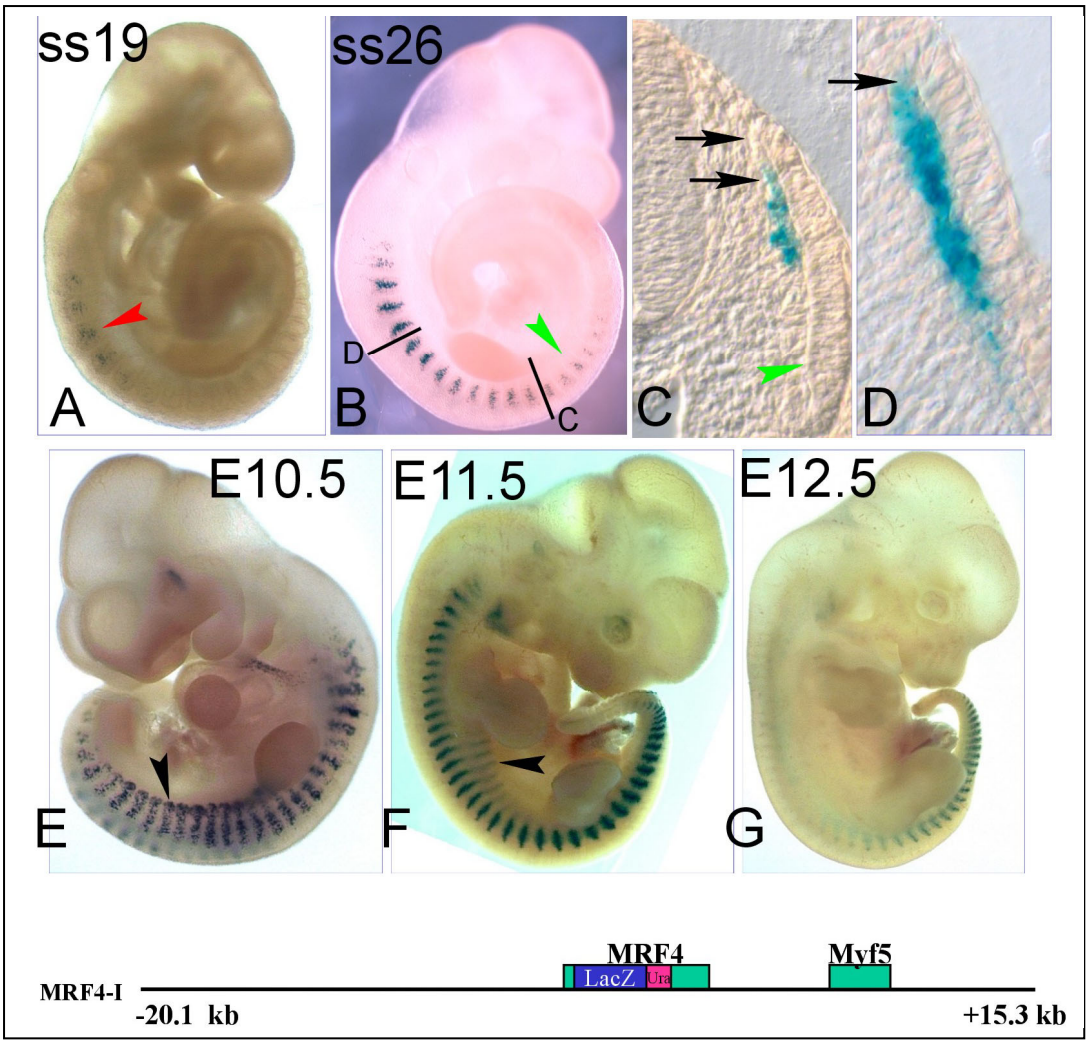

Figure 4. Expression of the largest transgene MRF4-1 illustrated by LacZ staining. Expression starts at ss19 in the dorsal half of cranial somites (A, marked by red arrowhead) and proceeds during subsequent development rostro-caudally along the body axis as well as in the dorso-ventral direction within somites (A-F). Transverse section of E9.5 illustrates expression in central myotome of young somites sparing the most dorsal region (black arrows in C). In more mature somites expression extends further dorsally and ventrally (D). Levels of sections are indicated by lines in panel (B). The early ventral domain is not detected with the transgene (green arrowheads in (B) and (C). At E10.5 and E11.5 expression is seen also in the hypaxial part of thoracic somites (black arrowheads in (E) and (F). At E12.5 expression is down-regulated in cervical and thoracic somites (G).

3.1.4. The upstream region –20.1/ -11.4 kb of the MRF4 gene is dispensable for its expression in somites

Expression of the MRF4-I transgene in somites essentially recapitulated the full pattern of the endogenous MRF4 gene. In order to localize elements responsible for MRF4 expression in specific compartments of somites, further analysis of the 5'-end sequence was performed. 6.1 kb and 2.6 kb fragments were successively removed from the 5'-end of the MRF4-I construct using single-cutting restriction enzymes. The resulting transgenes, MRF4-II and MRF4-III encompassed -14/+15.3 kb and -11.4/+15.3 kb sequences, respectively. Comparative BLAST analysis of sequences of the deleted fragments with the human genome revealed short regions with significant homology to those in the corresponding region of the human MRF4 locus (Fig. 5 D). Recent studies demonstrated a role of such conserved non-coding sequences in gene regulation (Buchberger et al., 2003; Summerbell & Rigby, 2000). However, the expression patterns of MRF4-II and MRF4-III containing 14 and 11.4 kb sequence upstream of the MRF4 gene, respectively, were found undistinguishable from that of MRF4-I carrying 20.1 kb upstream sequence (Fig. 5 and Fig. 6). Both constructs were expressed in somites at early developmental stages starting in the dorsal part of the myotome (Fig. 5 A; Fig. 6 A, B). Expression then progressed in rostro-caudal and dorso-ventral directions. Transverse sections of mature somites demonstrated the expression of MRF4-III transgene in the epaxial, central and hypaxial myotome (Fig. 6 D, F, I).

The sections through younger somites illustrated that the transgene was particularly strong in the central part of the myotome where nuclei tend to accumulate (Fig. 6 G). Thus, the –20.1/ -11.4 kb region despite the sequences conservation during evolution appears dispensable for the MRF4 regulation in somites, at least in this transgenic approach.

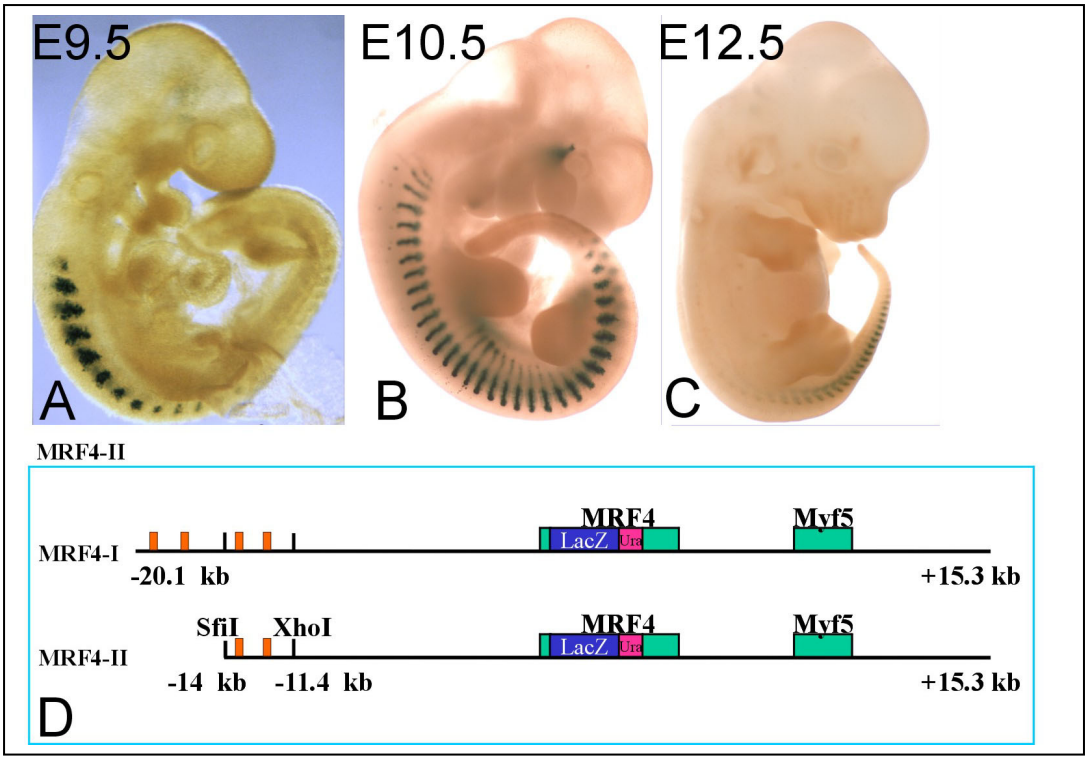

Figure 5. **Whole-mount LacZ staining of embryos carrying the MRF4-II transgene (A-C).** Expression is first detected in somites at E9.5 (A). At E10.5 the transgene is expressed in the myotome of all somites (B). Thoracic somites display the complete dorso-ventral pattern. Expression is down-regulated in rostro-caudal direction at E12.5 (C). (D) demonstrates schematically the constructs for MRF4-I and MRF4-II. Orange boxes represent homology with human genome.

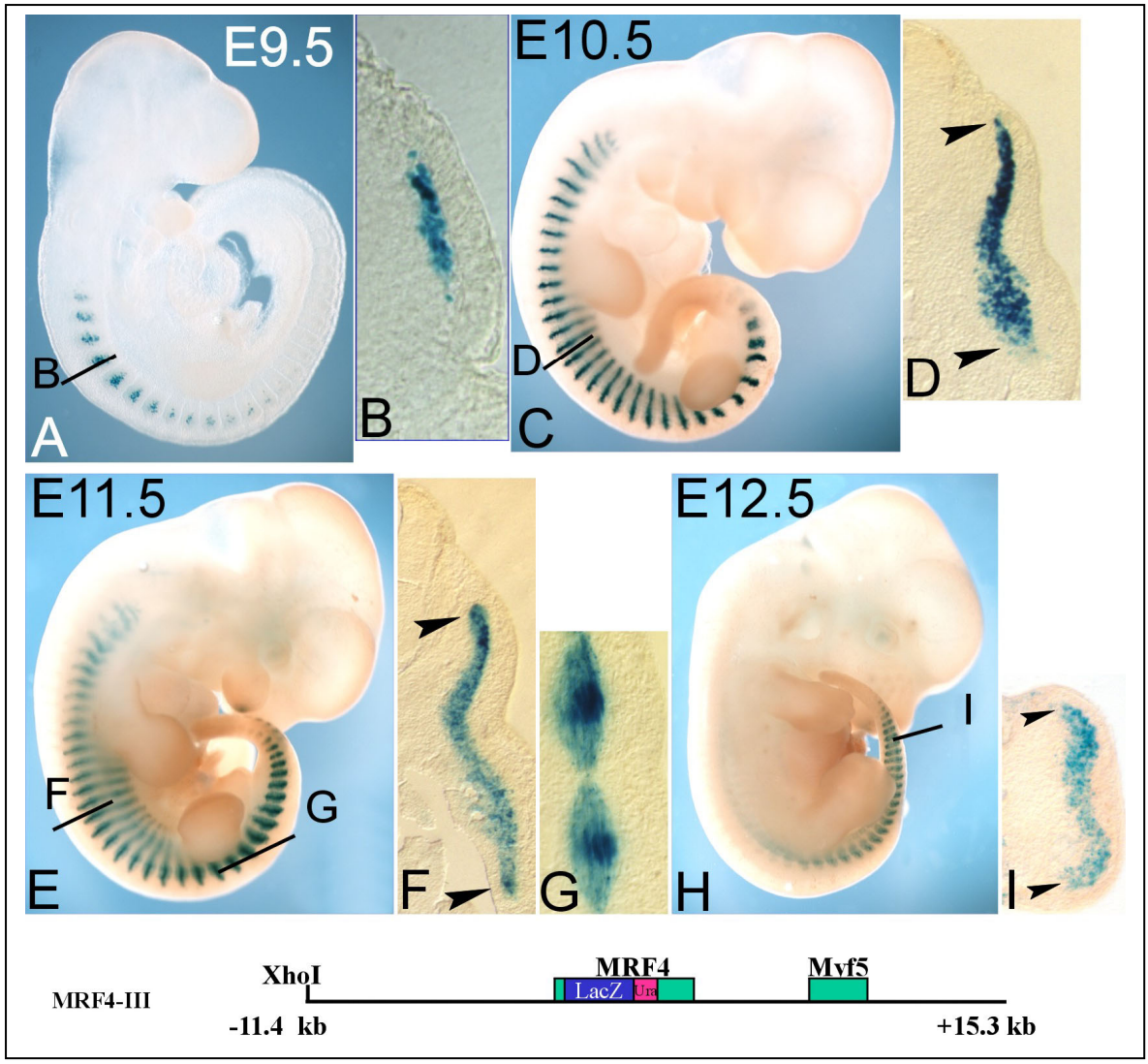

Figure 6. Expression pattern of the MRF4-III transgene illustrated by LacZ staining. Expression starts at E9.5 (A and B). At E10.5 (C), E11.5 (E) and E12.5 (H) expression is observed in full dorso-ventral extent of somites (D, F and I, upper and lower black arrowheads indicate the epaxial and the hypaxial domain, respectively). The frontal section on hind limb level (G) shows that the transgene is particularly strong expressed in the central part of the myotome. Some nuclear leakage reveals the longitudinal orientation of spindle-shaped myocytes. The planes of sections are indicated by lines on the adjacent panels.

3.1.5. The downstream sequence between +3.9 kb and +15.3 kb of the MRF4 gene can be removed for transgene expression in somites

In construct MRF4-IV, the +3.9/+15.3 kb sequence downstream of the MRF4 gene was removed at the SpeI site to evaluate the potential influence of this region. The deleted sequence encompassed the Myf5 gene and several of the associated enhancers. These included the branchial arch, the central nervous system enhancer, and the hypaxial regulatory element situated in the body of the Myf5 gene (Summerbell et al., 2000). Like the previous constructs, the MRF4-IV transgene was expressed in all somites of transgenic embryos, suggesting that the downstream sequence is not necessary for most aspects of MRF4 expression in somites (Fig. 7 A-D). However, the hypaxial extension of the expression domain in thoracic somites was substantially more variable with this construct than with MRF4-I, MRF4-II or MRF4-III (Fig. 7 B, C) indicating that additional elements within the removed 3'-end may contribute to stabilize expression in the hypaxial domain. The early epaxial enhancer regulating Myf5 expression in the more dorsal domain including dermomyotome was still present in this construct. Nevertheless, transgene expression was activated correctly in the myotome and was not observed in the very dorsal dermomyotome (Fig. 7 A). Hence, it seems that this Myf5-specific enhancer does not activate the MRF4 promoter even in the context of the MRF4-IV transgene.

3.1.6. An important control region for MRF4 expression in the myotome is located between –7.2 kb and –3 kb

In order to assess the potential role of the 5'-upstream region in MRF4 regulation, the -11.4/-3 kb sequence was truncated to the PmeI restriction site at –3 kb in the MRF4-V construct. Analysis of 18 transient embryos and four stable lines did not reveal appreciable expression at any developmental stage (see Table 1). Two out of four stable lines showed only faint LacZ-staining in few myotomal cells of the cervical somites (Fig. 7 E, F). These together with previous results indicate that an essential gene control region has been removed in this construct.

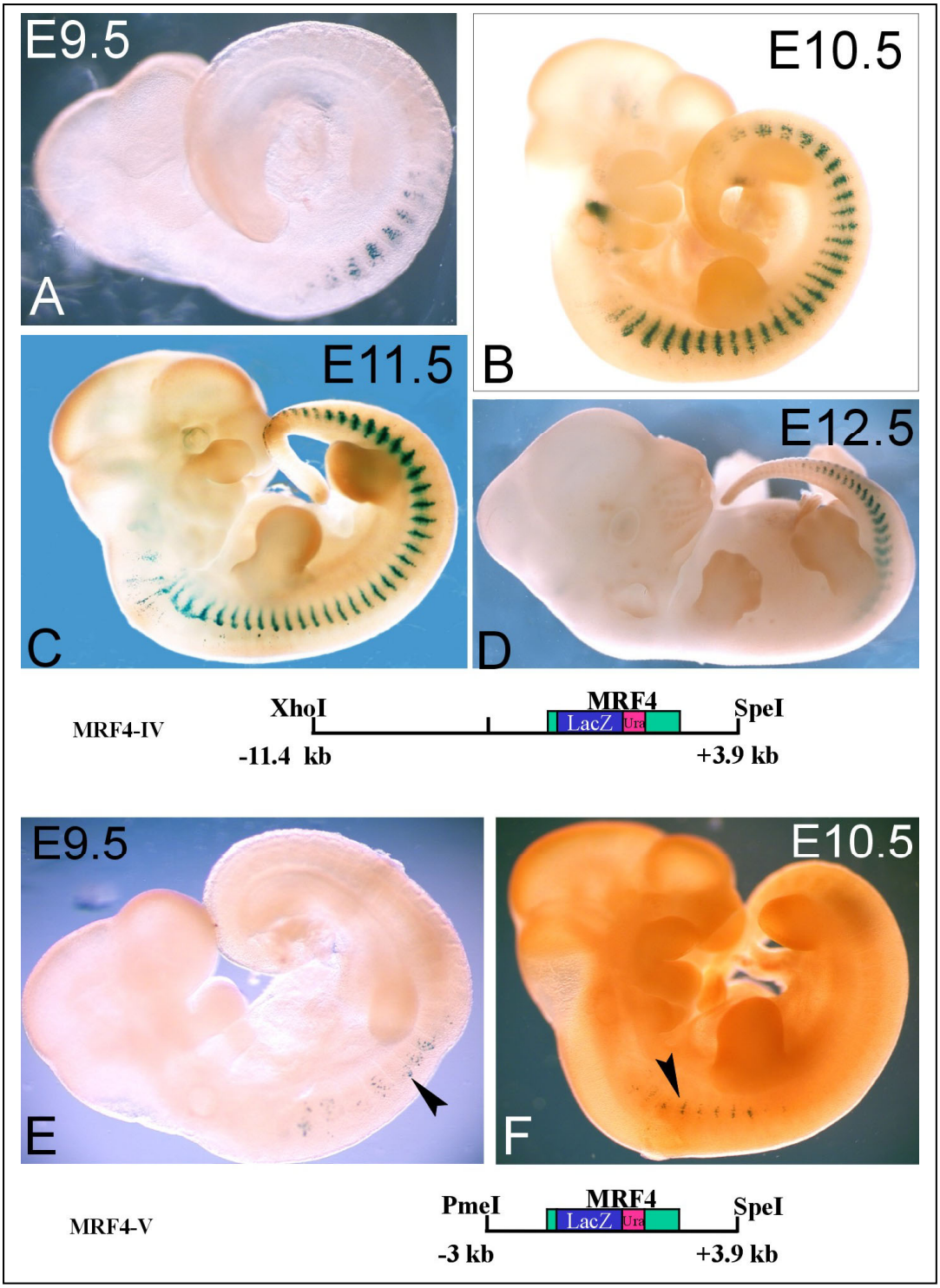

Figure 7. Whole-mount LacZ staining of embryos carrying the MRF4-IV and MRF4-V transgenes. MRF4-IV transgene in which the 3'-sequence was truncated to 3.9 kb downstream of the MRF4 gene was expressed in all myotomes of transgenic embryos (A-D). In contrast to that, MRF4-V containing the 3-kb promoter fragment drives only very faint expression in cervical somites (E, F and arrowheads there).

Considering the transgenes examined so far, one can conclude that essential regulatory elements for MRF4 expression in myotomes are located between –11.4 and –3 kb. Since MRF4-V contained the proximal promoter but was unable to drive significant expression in myotomes, this construct was used as a "minimal promoter" in combination with other sequences of the locus to test their activity in transgenic animals.

In order to further dissect regulatory elements within the -11.4/-3 kb region, about half of this sequence was deleted. The proximal part of the region was employed in two constructs, MRF4-XI and MRF4-XII containing 5'-upstream sequences to –7.5 kb and –7.2 kb, respectively. In contrast to MRF4-XI extending 3.9 kb downstream of the gene to a SpeI site, MRF4-XII contained only 3 kb downstream sequence up to a BamHI site (Fig. 8 K). Both transgenes generated essentially the same spatiotemporal expression pattern during somite development, as it was shown for MRF4-XII in Fig. 8 (A-D). These data suggest that the region -11.4/-7.2 kb is not required for MRF4 expression in somites, whereas the sequence between -7.2 and -3 kb contains important regulatory elements.

3.1.7. Myotomal expression of the MRF4 gene can be delimited to a 1-kb core element.

To further dissect the 4.2-kb sequence (-7.2/-3.0 kb) containing the putative myotomal MRF4 regulatory elements, various deletions were introduced into the 7.5-kb promoter fragment. In the construct MRF4-VII a PCR fragment from –7.5 to –5.6 kb was directly linked to the –3/+3.9 kb promoter fragment (the construct MRF4-V), thereby deleting the 2.6 kb between -5.6 kb and -3 kb while the entire promoter sequence shortened at the BglII site up to –5.6 kb was employed in transgene MRF4-VIII (Fig. 9 F).

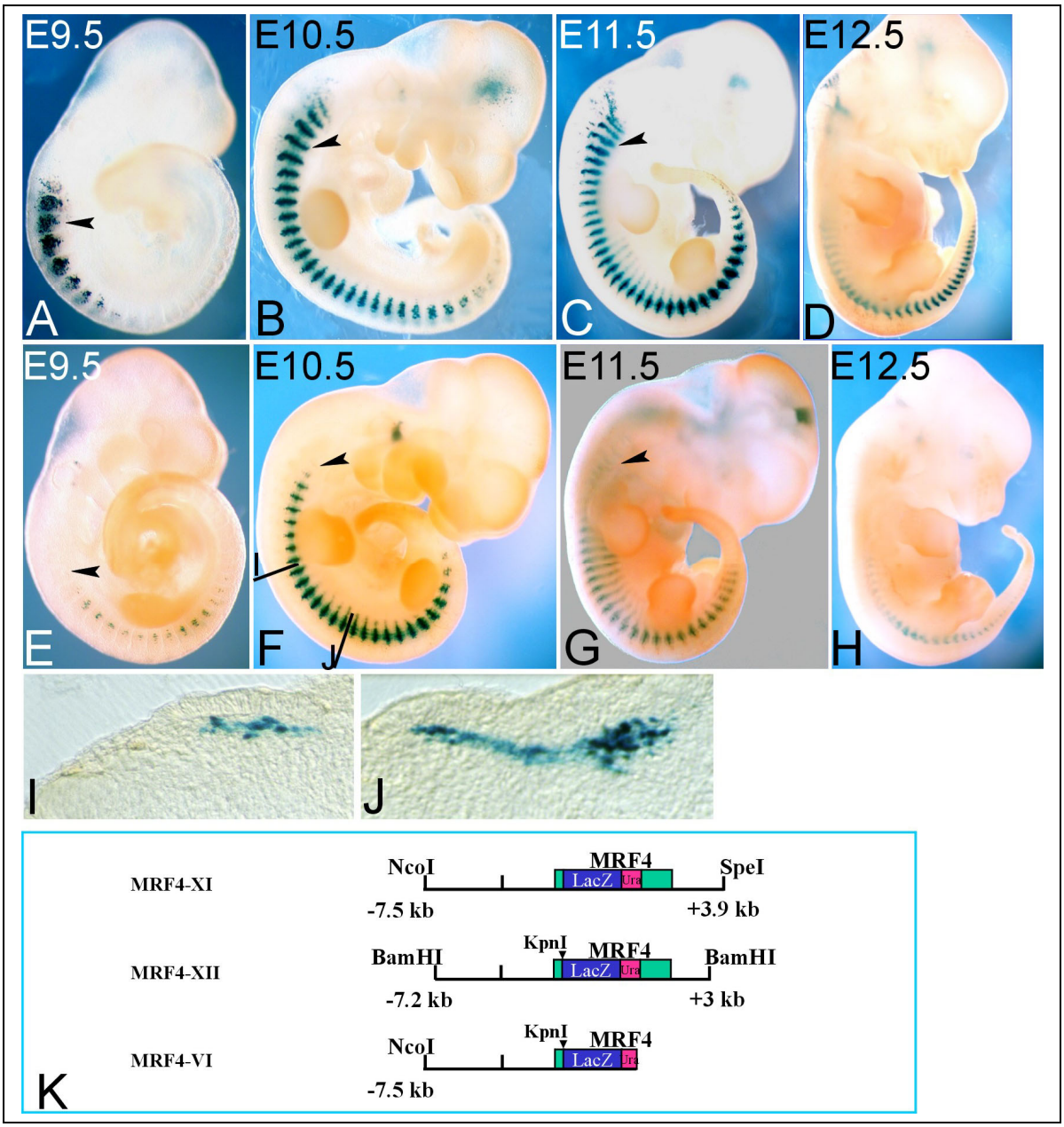

Figure 8. Expression patterns of MRF4-VI and MRF4-XII transgenes. LacZ staining reveals expression of the MRF4-XII (A-D) and MRF4-VI (E-J) transgenes in the myotomes. Hypaxial expression in the thoracic somites is slightly variable. A truncation of the 3'-end fragment including most of the gene body does not affect significantly expression in thoracic and tail somites between E10.5 and E12.5, except of some variations in intensity of staining (B-D versus F-H). However, the deleted fragment is essential for expression in rostral cervical somites (indicated by arrowheads in F-H, comparing to B-D). At E9.5 expression of MRF4-VI (E) is significantly weaker than that of MRF4-XII (A). Transverse sections of somites at E10.5 (the planes of sections are indicated by lines in (F) confirm specific transgene expression in the myotome (I, J). A schematic representation of MRF4-VI, MRF4-XI, and MRF4-XII constructs is shown in panel K. The construct MRF4-XI exhibits expression identical to MRF4-XII (data not shown).

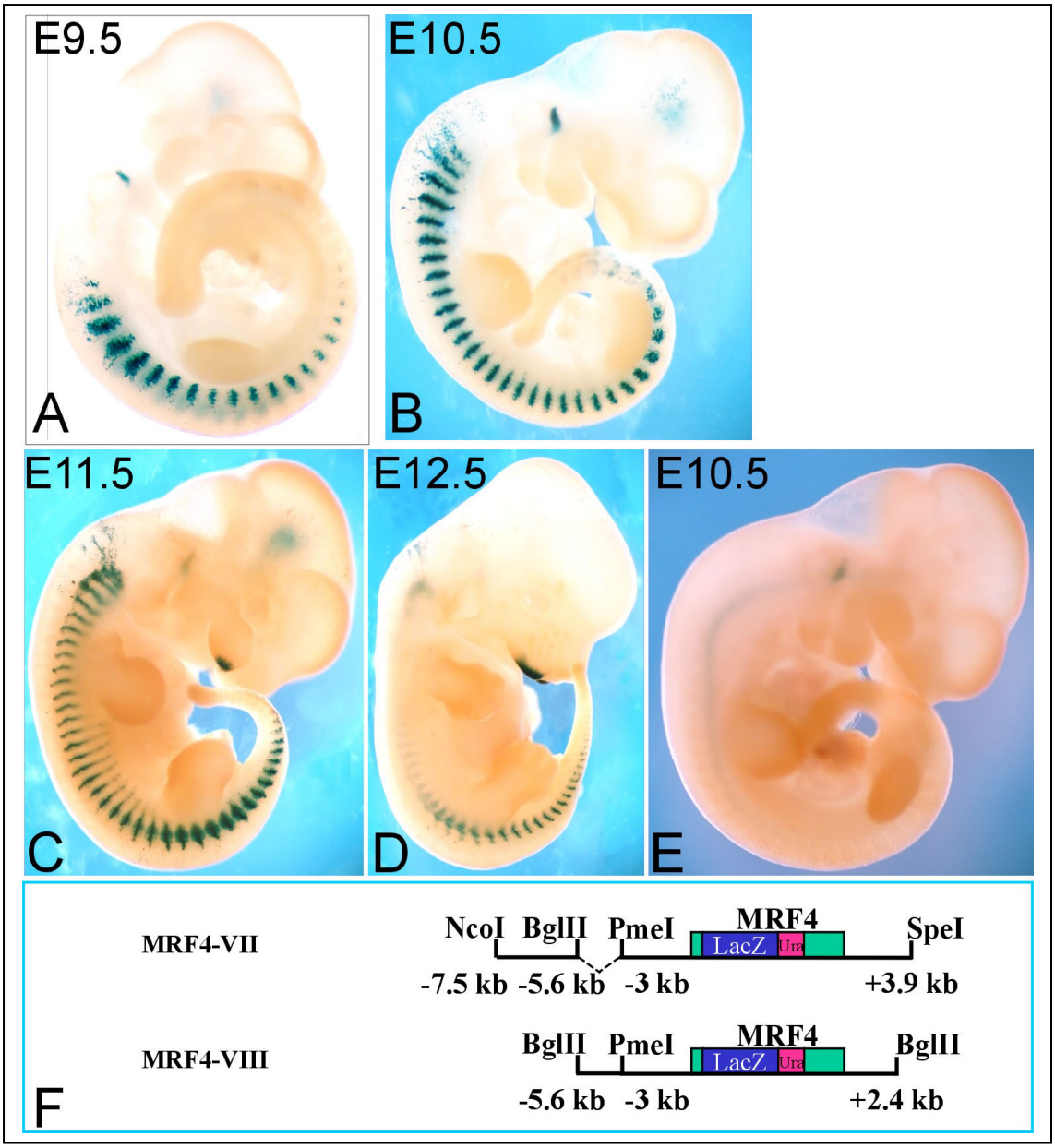

Figure 9. Expression patterns of the MRF4-VII and MRF4-VIII transgenes. Whole-mount β–galactosidase staining of MRF4-VII and MRF4-VIII transgenic embryos is shown. MRF4-VII carries an internal deletion from –5.6 to –3 kb within the 7.5-kb MRF4 promoter fragment and generates the appropriate pattern in myotomes of all somites (A-D). In contrast, 5' truncation from -7.5 to -5.6kb in the construct MRF4-VIII leads to ablation of transgene expression in all somites (E). The schematic representation of constructs MRF4-VII and MRF4-VIII is shown (F).

Significantly, expression of the MRF4-VII transgene started normally in cranial somites around E9.0 and expanded later into caudal direction (Fig. 9 A). As expected, at E10.5- E11.5 the transgene was expressed in all somites (Fig. 9 B, C). In contrast, embryos harboring MRF4-VIII transgene, which contains the proximal promoter region (-5.6 kb) failed to express the transgene in somites (see Table 1 and Fig. 9 E). From these results one can conclude that important control elements for expression in myotomes lie within the –7.5 to –5.6 kb sequence interval.

The region –7.5 to –5.6 kb, therefore, was further subdivided into two parts with an overlap of about 100 bp. These DNA fragments were generated by PCR and placed separately in front of the 3-kb promoter. MRF4-IX contained the distal fragment –7.5/-6.5 kb, while MRF4-X contained the proximal fragment (–6.6/-5.6 kb). Expression of MRF4-IX occurred only in cervical and tail somites (Fig. 10 A-C, E, and G). No expression was detected in the thoracic region (Fig. 10 F, I). Moreover, MRF4-IX was expressed in the dermomyotome of young somites (Fig. 10 H, J) where MRF4 is normally never expressed.

Expression of the MRF4-X transgene was detected in all somites along the anterio-posterior axis during the appropriate developmental period (Fig. 11). The transgene was robustly expressed in myotomes but expression in the hypaxial domain of thoracic somites was variable and ectopic expression in the dermomyotome of the two oldest somites and in adjacent mesenchyme was observed occasionally. These data provide evidence that the 1-kb fragment located at –6.6 to –5.6 kb upstream of the MRF4 gene in conjunction with the 3-kb promoter fragment correctly activates MRF4 gene expression in all somites along the body axis, while none of the other sequences tested with the MRF4 promoter (-3 kb) was able to support correct myotomal expression in the mouse embryo.

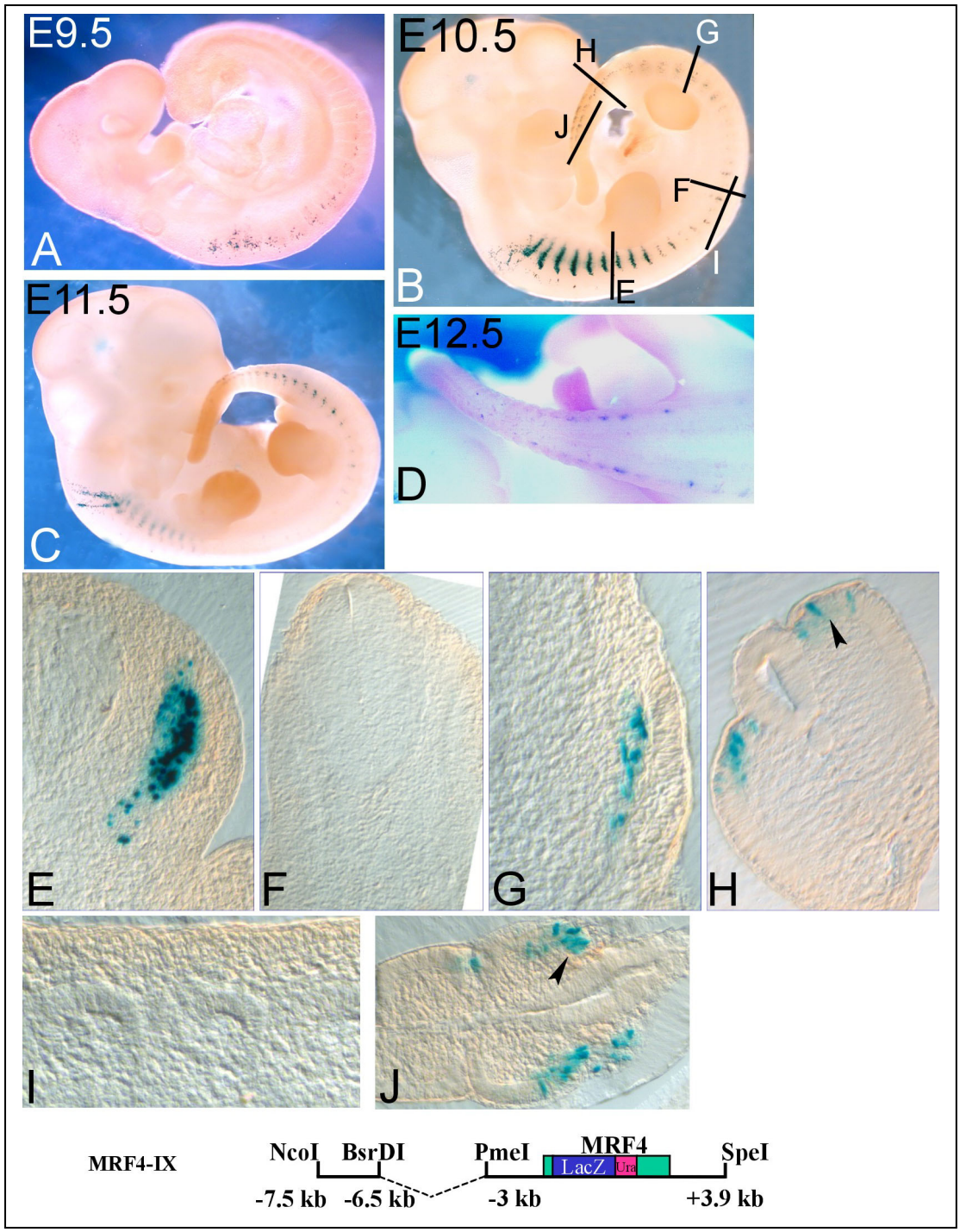

Figure 10. LacZ staining of embryos carrying the MRF4-IX transgene. The transgene containing 3-kb minimal promoter linked to –7.5/-6.5 kb sequence is expressed in cervical, lumbar and tail somites but not in the interlimb region (A-D). The planes of sections (E-J) of the LacZ stained E10.5 embryo are shown in panel (B). Transverse sections at fore and hind limb levels demonstrate myotomal expression (E, G). Transverse and coronal sections in the interlimb region reveal no expression (F, I). In the youngest somites expression occurs in the dermomyotome (shown by arrowhead in H and J).

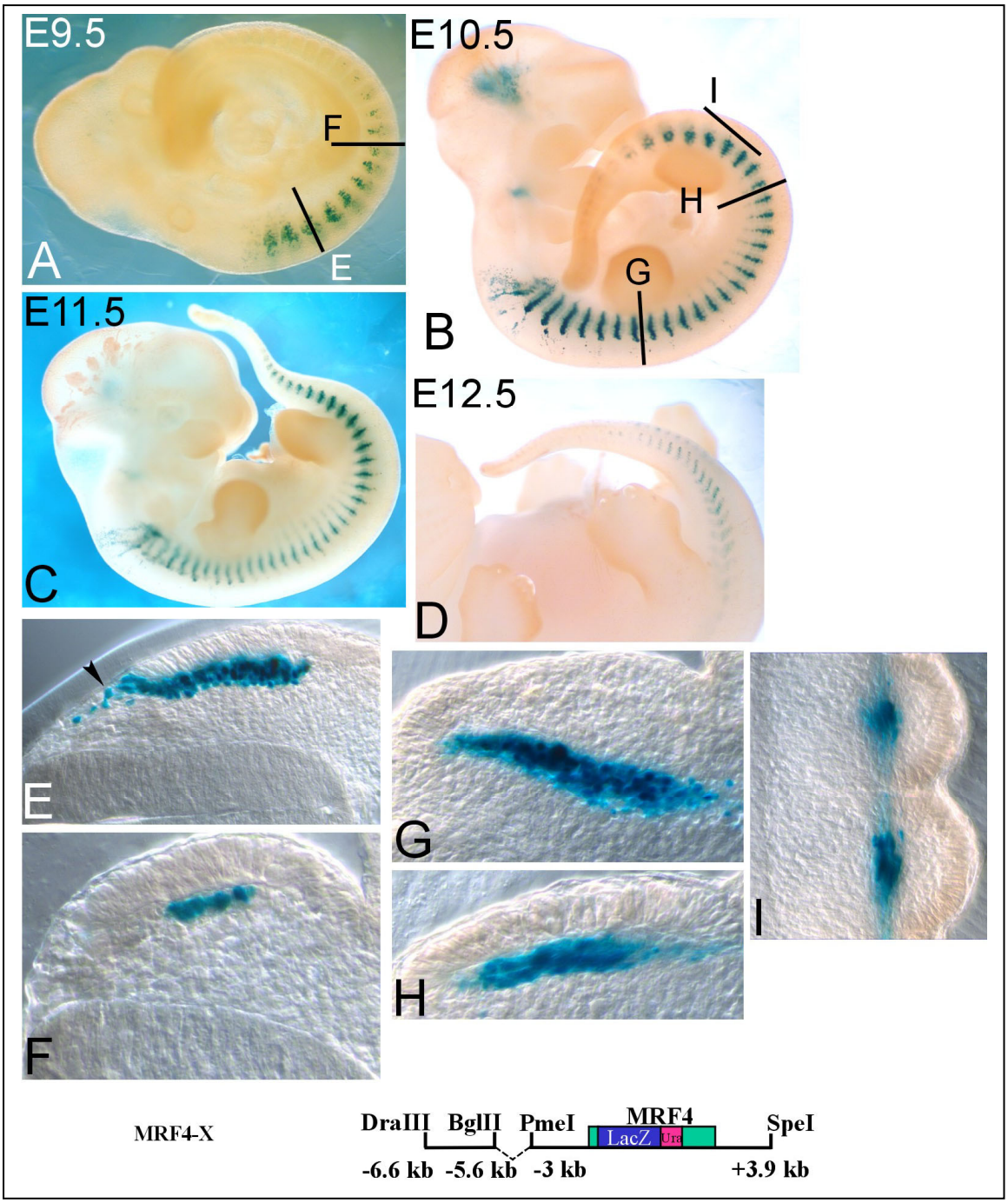

Figure 11. Expression pattern of the MRF4-X transgene assayed by LacZ staining. The transgene corresponding to the –6.6/-5.6 kb sequence linked to the 3-kb promoter fragment promotes myotomal expression in all somites along the body axis during the correct developmental time frame (A-D). The planes of sections are shown by lines in (A) and (B). In two/three oldest cervical somites expression is not restricted to the myotome but ectopically appears in the most dorsal part of the dermomyotome and in adjacent mesenchymal cells (arrowhead in E). In all other somites expression is restricted to the myotome (F). Transverse and frontal sections of E10.5 embryo demonstrate the proper myotomal localization of signal (G-I). In (E-H) dorsal is to the left.

Computer-based analysis of the 1-kb sequence (–6.6/-5.6 kb) using Genomatix and Transfac databases suggested about 500 potential binding sites for transcription factors. Among them were sites for factors with documented roles in the myogenic program, such as E-boxes, MEF2 binding sites, and homeodomain binding sites. Evolutionary Conserved Region (ECR) Browser analysis revealed a 360-bp sequence (-6.56/-6.2 kb) that was significantly homologous to the corresponding region of the human and dog MRF4 locus including a 117-bp region (-6.47/-6.35 kb) with high homology to the chicken MRF4 locus, while adjacent sequences were highly divergent in these species (Fig. 12), suggesting that the conserved sequence may represent a functional core element for MRF4 gene control.

3.1.8. The +0.2/+3 kb downstream sequence of the MRF4 gene contributes to its expression in the most rostral cervical somites

Patapoutian and colleagues reported that an MRF4 transgene construct containing a 6.5 kb promoter fragment to the upstream BamHI site (a 7.2 kb fragment according to the genomic sequence) was not expressed in somites (Patapoutian et al., 1993). This construct lacked all sequences downstream of the MRF4 gene. The MRF4-XII construct, used in the present study, also contained the same 5'- BamHI fragment and was expressed in somites. Since the main difference between Patapoutian's construct and MRF4-XII was the additional 3-kb downstream sequence including the gene itself, it appears that this region may play a critical role for MRF4 expression in somites. To test the importance of this sequence it was removed in construct MRF4-VI, which maintained the intact 7.5 kb at the 5'- end (NcoI site). In the Patapoutian's construct the reporter gene was introduced into the SalI site, while in MRF-VI nLacZ was inserted into the KpnI site, resulting in additional 169 bp of MRF4 exon 1 as a part of this construct.

Transgene MRF4-VI was expressed in the myotome at E9.5- E12.5 (Fig. 8 E-J). This result was reproducible and has been observed in a number of transient embryos and in two stable mouse lines (Table 1). Hence, I suggest that elements responsible for most aspects of MRF4 expression in the myotome are located upstream of the gene. However, expression of MRF4-VI was generally

weaker than MRF4-XII at E9.5 (Fig. 8 E). Moreover, MRF4-VI expression was not seen in most rostral cervical somites, whereas MRF4-XII was expressed in all seven somites located rostrally to the forelimb buds (Fig. 8 compare F with B and G with C). Taken together, these data suggest that the sequence between +0.2 and +3 kb encompassing the MRF4 gene itself contributes to elevated expression level during the early developmental period and also regulates expression in the most cranial somites. It should be noted, however, that this sequence alone cannot drive myotomal expression without additional upstream regulatory elements, as the failing activity of MRF4-V clearly demonstrated.

The constructs MRF4-I to MRF4-XII for technical reasons contained the yeast Ura3 gene that was used as a selection marker in the original YAC construct (Zweigerdt, 1998). To rule out that the yeast Ura3 gene including its promoter influences the expression pattern of the transgenes it was completely removed in the construct MRF4-XIV. This transgene generated essentially the same pattern as the Ura3 gene containing construct MRF4-X, although the level of LacZ staining appeared generally weaker (Fig. 13). Some variation of expression intensity was observed, however, with all transgenes used in this study suggesting that copy number and site of integration can exert some effect on expression level, independent of the presence or absence of the yeast Ura3 gene. Interestingly, MRF4-XIV lacks most of the gene body and 3'- downstream sequence (similar to construct MRF4-VI), and was not expressed in the most rostral somites. This observation supports the idea of intragenic or 3'-downstream sequence playing a role in controlling MRF4 expression in rostral somites.

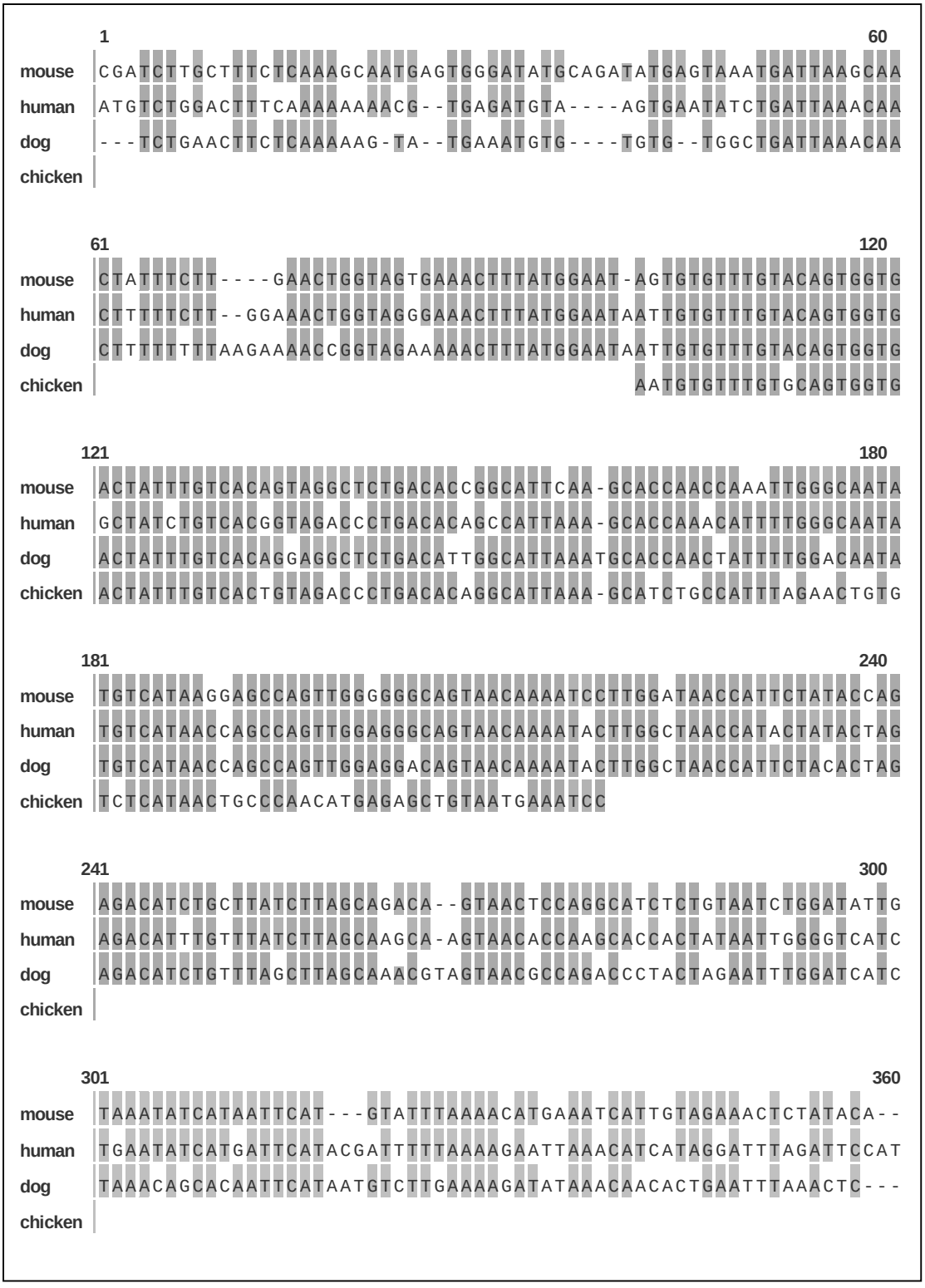

Figure 12. **A block of 360 nucleotides at –6.5 kb upstream of the mouse MRF4 gene is highly conserved in the corresponding region of the human and dog MRF4 locus**. This sequence includes a 117-nucleotide region, which is also conserved in the chicken MRF4 locus. Sequence alignment was performed by the Clustal W method. Identical nucleotide residues are shadowed by gray boxes. Nucleotide gaps are marked by dashes.

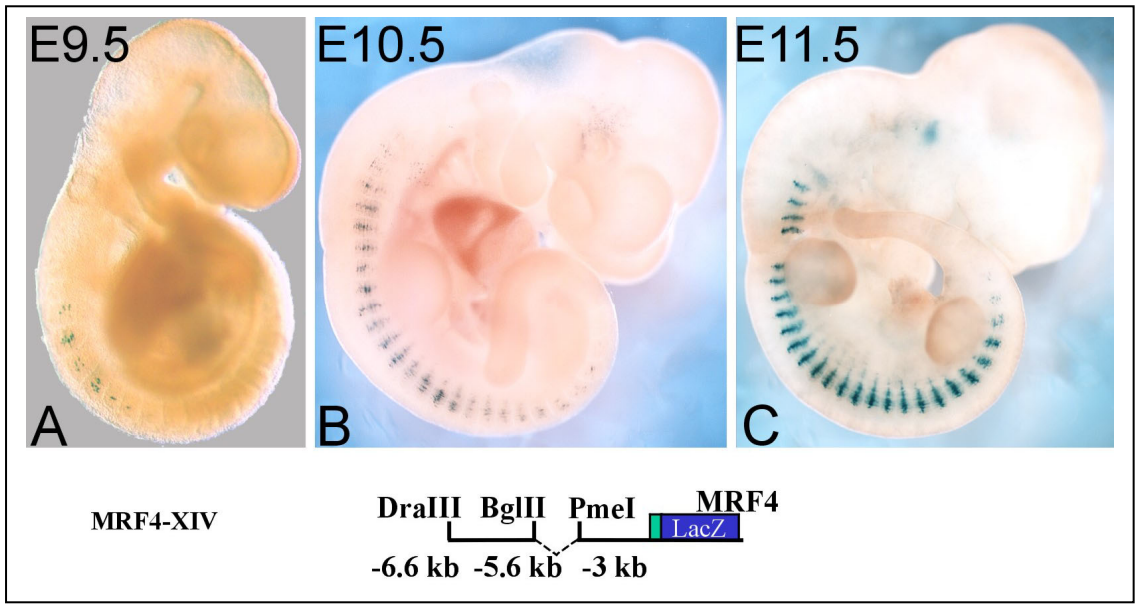

Figure 13. Expression pattern of MRF4-XIV. This construct is identical to MRF4-X except that it lacks the 3' portion of the gene and does not contain the yeast Ura3 gene. Expression assayed by whole-mount LacZ staining of transgenic embryos appears in all myotomes of transgenic embryos except of the upper cervical somites (A-C) but somewhat weaker comparing to MRF4-X expression.

3.2. Control regions involved in MRF4 expression in the fetus and the adult mouse

3.2.1. The region between +3.9 kb and +15.3 kb downstream of the MRF4 gene contributes to expression in fetal and adult muscles

It was demonstrated that the regulation of early myotomal expression is controlled by separate elements from those controlling late fetal expression (Patapoutian et al., 1993; Pin et al., 1997; Pin & Konieczny, 2002). In order to investigate elements regulating late MRF4 expression in muscles, all stable transgenic lines produced in this study were examined at different fetal stages and in adult mice. Some of the constructs were also tested in transient embryos at E14.5 and E17.5. The results are summarized in Table 2.

Table 2. Summary of transgenic embryos expressing β-galactosidase in fetal and adult muscles. Stable lines were examined at different stages between E14.5 and newborn, and in some cases in adulthood. For constructs MRF4-IV and MRF4-XIII, transient embryos at E14.5 and E17.5, which failed to express the reporter gene but contained the transgene, are also listed. Numbers in parenthesis refer to total number of transient embryos and lines examined at different stages. [a] expression in all or most of fetal muscles. [b] expression in the semispinalis cervicis m. [d] in these two lines the transgene was also expressed in lumbar muscles. [c] Variable ectopic expression was observed mostly in the neural tissue and rarely in the cartilage.

Transgene	Transient E14.5 or E17.5	Line	Fetal muscle expression		
			Complex muscle pattern [a]	Partial muscle pattern [b]	Ectopic [c]
MRF4-I	-	1	1(1)	-	-
MRF4-II	-	1	1(1)	-	-
MRF4-III	-	1	1(1)	-	1(1)
MRF4-IV	5	3	1(8)	2(8) [d]	1(8)
MRF4-V	-	4	1(4)	-	-
MRF4-VI	-	2	0(2)	-	-
MRF4-VII	-	3	0(3)	1(3)	-
MRF4-VIII	-	1	0(1)	-	-
MRF4-IX	-	4	0(4)	-	-
MRF4-X	-	2	0(2)	1(2)	-
MRF4-XI	-	3	1(3)	1(3)	1(3)
MRF4-XII	-	2	0(2)	1(2)	-
MRF4-XIII	4	-	0(4)	-	-

It was demonstrated previously that the –7.2 kb sequence upstream of the mouse MRF4 gene is able to drive expression of the reporter gene in subset of fetal muscle (Patapoutian et al., 1993). As most transgenes used in this study encompassed the –7.2 kb upstream region, it was expected to observe expression in fetal muscles with these transgenes.

Transgenic lines carrying transgenes MRF4-I, MRF4-II, and MRF4-III expressed the transgenes in fetal and adult muscles (Fig. 14 and Fig. 15) Expression started at E14.5 and intensity of LacZ staining increased with further fetal development, in good agreement with the endogenous MRF4 expression pattern (Bober et al., 1991). In Fig. 14, expression of the transgene in the MRF4-I line 1 is shown in head and neck muscles at E16.5 and E17.5 (Fig. 14 A-C). Fig. 14, D-H represents expression of the transgene in the MRF4-II line 1 at E15.5. LacZ staining is shown for head, shoulder, and trunk muscles (Fig. 14 D-H). Details of the muscle expression pattern at different developmental stages are shown for the shorter transgene MRF4-III in Fig. 15.

The first wave of MRF4-III expression in somites ends at E12.5- E13.0 (Fig. 6 H). Transgene expression reappeared at E14.5 in the tongue, extraocular muscles, rostral muscles of back and in the neck (Fig. 15 A-C). Expression then rapidly expanded into all muscle forming regions and at E15.5 – E16.5 it was observed in most trunk and limb muscles (Fig. 15 D). At E17.5 all muscles expressed the transgene (Fig. 15 E-I and data not shown). Expression was maintained at high level in all examined muscle fibers of adult mice. As demonstrated by histological analysis of adult muscle in the MRF4-III line 1, the transgene was expressed in typical slow, as soleus m. (Fig. 15 N), typical fast, as long extensor m. of digits (Fig. 15 K) and tibialis anterior m. (Fig. 15 J, O), and mixed muscles, as gastrocnemius (Fig. 15 L) and femoral quadriceps (Fig. 15 M). The oldest tested animals were one year old.

Therefore, these results suggest that elements sufficient for the fetal and adult phase of MRF4 expression are located in the region between –11.4 kb and +15.3 kb.

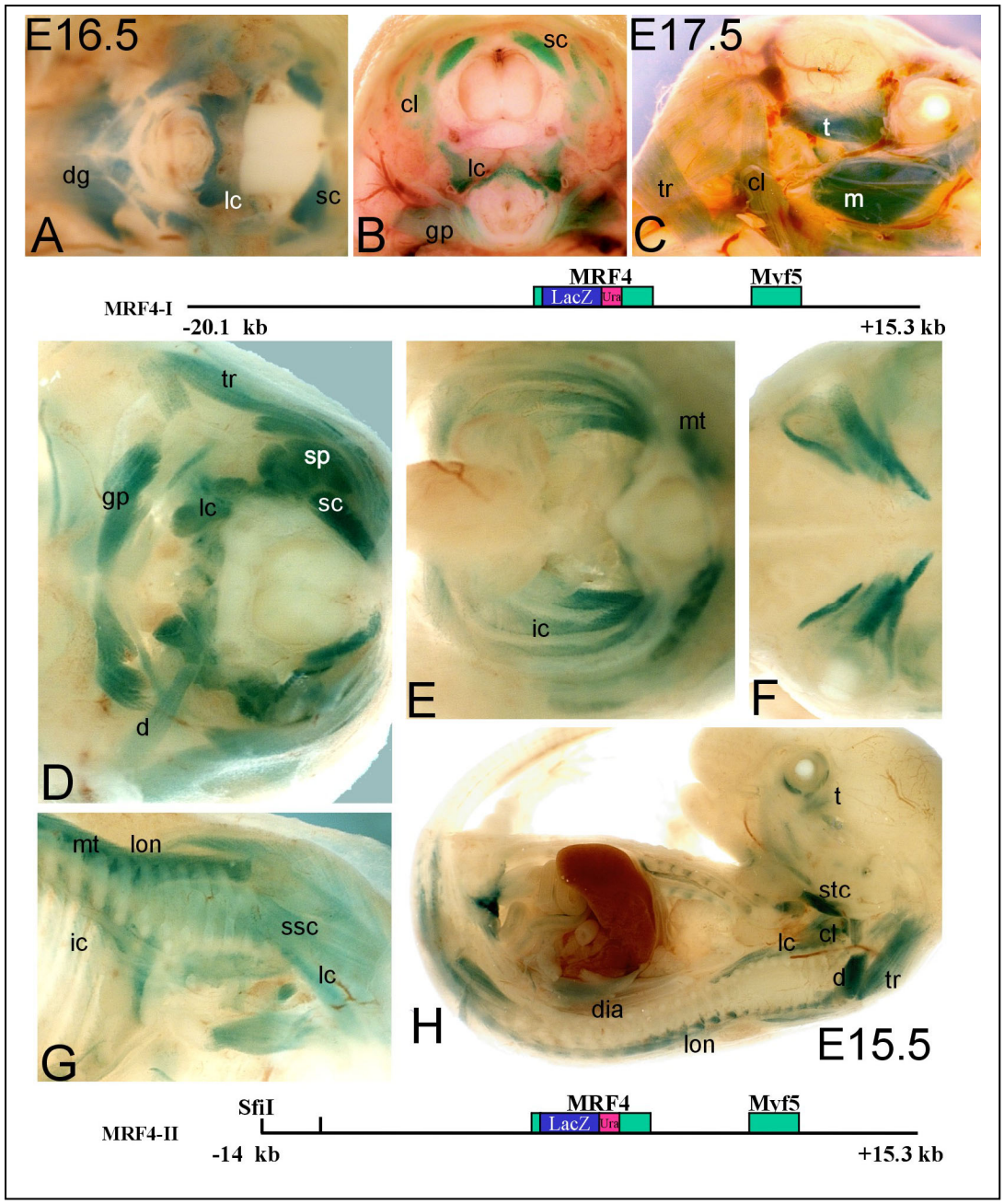

Figure 14. LacZ staining reveals expression of the MRF4-I and MRF4-II transgenes in fetal muscles. Expression of MRF4-I line-1 at E16.5 (A, B) and E17.5 (C) is shown in neck, shoulder and in head muscles. Panels (A) and (B) are transverse sections (caudal and rostral view, respectively). The panel (C) is a lateral view of the head. Expression of MRF4-II line-1 is demonstrated at E15.5 on transverse sections (D, neck level, rostral view, and E, abdominal level, caudal view), dorsal section (F, dorsal view) and sagittal sections (G, H). Muscles of neck and shoulders (D, G, H), trunk (E, G, H), and head (D, H), including the extraocular muscles (F) are stained. Abbreviations: cl- cleidocephalic; d- deltoid; dg- digastricus; dia- diaphragm; gp- greater pectral; ic- intercostal; lc- longus cervicis; lon- longissimus (cervicis or thoracis); m- masseter; mt- multifidius thoracis; sc- semispinalis cervicis; sp- splenius capitis; ssc- supscapularis; stc- strenocleidocephalic; t- temporal; tr- trapezius.

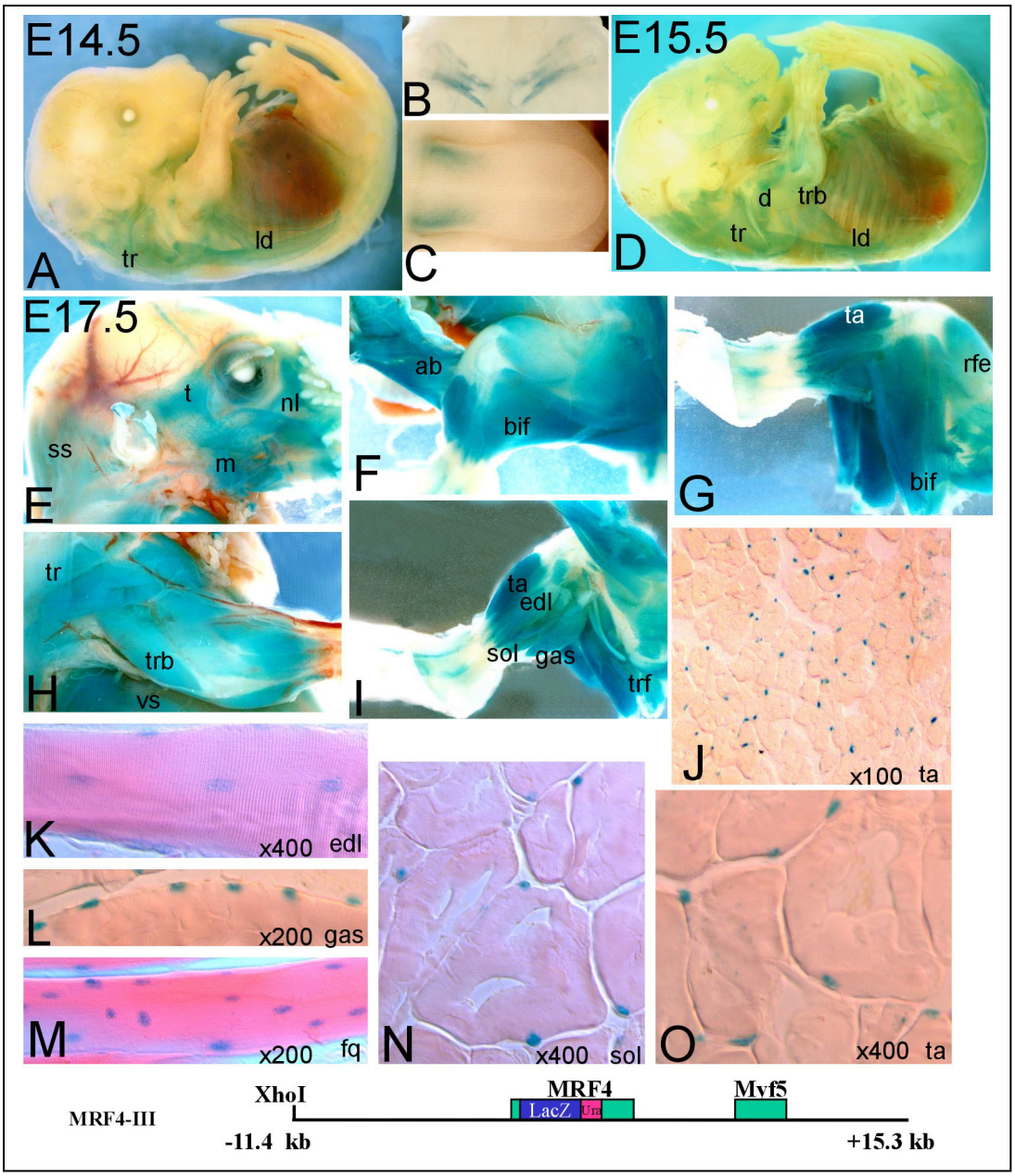

Figure 15. Expression of the MRF4-III transgene in fetal and adult muscles illustrated by LacZ staining. Expression starts at E14.5 in neck and upper back muscles (A), extraocular muscles (B) and in tongue (C). At E15.5 most trunk, limb and head muscles express the transgene (D). At E17.5 all muscles are stained. It is shown for head (E), forelimb (H), shoulder (H), hindlimb (F, G, and I- here biceps has been removed to expose the deeper located muscles) and the muscles of abdominal wall (F, ab). Frozen sections demonstrate LacZ-positive fibers in different adult muscle types (J-O). Transverse sections are shown in (J, N, O), and longitudinal sections are in panels (K-M). Abbreviations: ab- straight and oblique abdominal, bif- biceps femoris; d- deltoid; edl- long extensor m. of digitis; fq- femoral quadriceps; gas- gastrocnemius; ld- latissimus dorsi; m- masseter; nl- nasalabial levator; rfe- rectus femoris; sol- soleus; ss- superficial sphincter m. of the neck; t- temporal; ta- tibialis anterior; tr- trapezius; trb- triceps brachii; trf- triceps femoris; vs- ventral serrated.

In contrast to extensive muscle-specific expression detected in the mice carrying transgenes with the +15.3 kb downstream sequence, analysis of transgenes missing the +3.9/+15.3 kb sequence downstream of the MRF4 gene revealed essentially no transgene expression in fetal muscles. Two transgenes MRF4-IV and MRF4-XIII carrying -11.4/+3.9 kb and -11.4/+0.2 kb sequences, respectively, were analyzed in transient embryos at E14.5 and E17.5 and in stable lines. All of these transient embryos failed to express the reporter gene (see Table 2). One stable line showed weak expression in most of trunk and proximal limb muscles (Fig. 16 A-F) and two others demonstrated expression only in a single neck muscle and in the lumbar part of paravertebral muscles (Fig. 18. A-D).

Similarly, analysis of the other transgenes encompassing various upstream sequences but lacking the +3.9/+15.3 kb downstream region revealed that most of them were unable to express the reporter gene in fetal muscles (see Table 2). Of more than twenty examined only two stable lines exhibited weak expression in fetal trunk muscles (Fig. 16 G, H, and data not shown) and four stable lines showed expression in a single neck muscle, m. semispinalis cervicis (Fig. 17 E-H). This partial expression pattern of various transgenes in individual muscles might be due to position effects of the integrated construct. Clearly, the tested sequences upstream of the gene were not sufficient to support consistent expression in fetal muscles. Therefore, it seems that critical elements for stable MRF4 expression in fetal muscles are located in the +3.9/+15.3 kb downstream region.

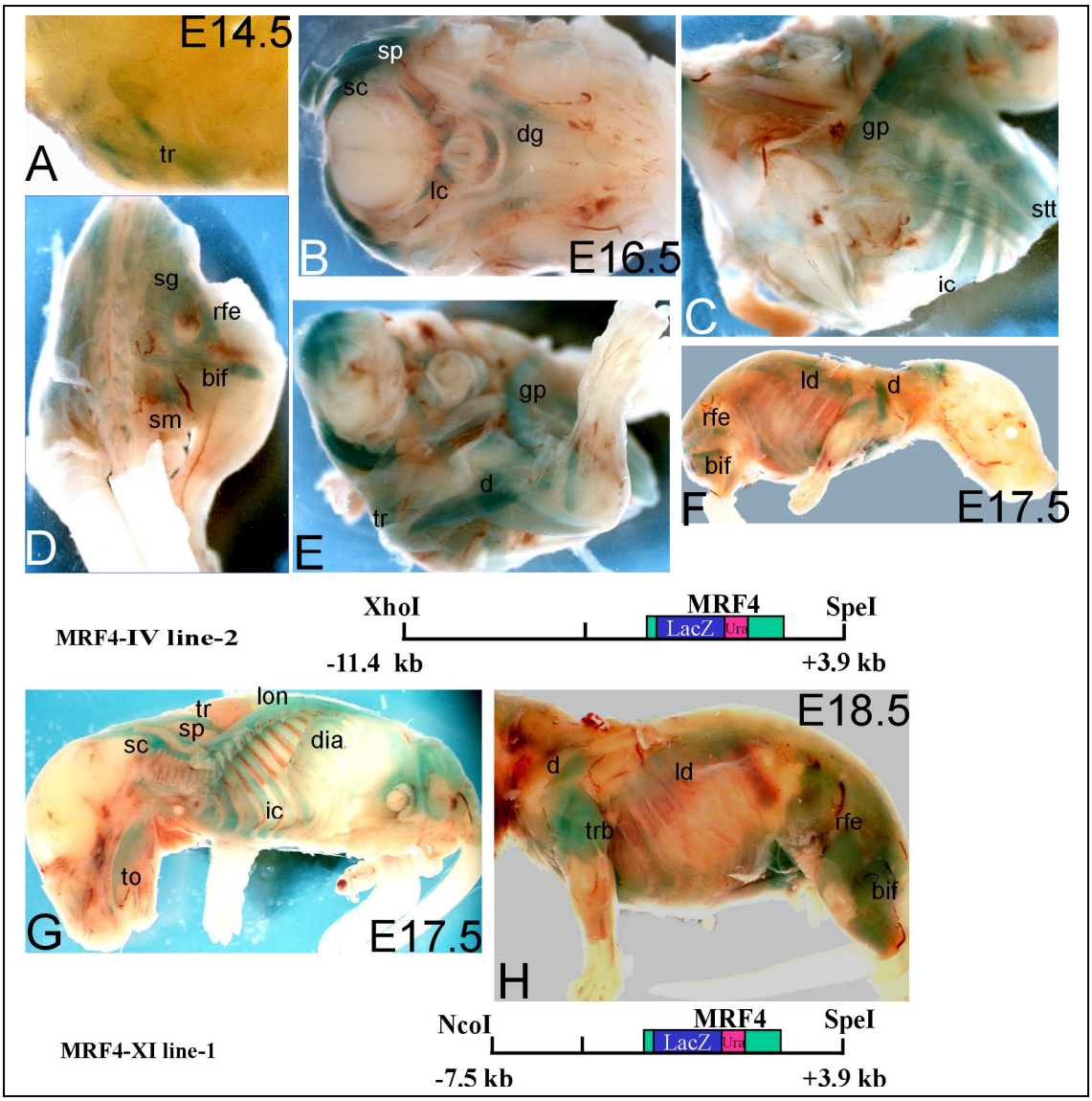

Figure 16. LacZ staining in fetal muscles of the MRF4-IV line-2 and the MRF4-XI line-1 transgenic embryos. LacZ-staining in the MRF4-IV line-2 embryos appears at E14.5 (A, fragment of the neck). At E16.5 expression is detected in head (B, transverse section, caudal view), in neck, in shoulders (E), in forelimbs (E), in chest (C, view of the ventral surface), in back and in hindlimbs (D). Lateral view of the E17.5 embryo demonstrates partial staining in limbs, neck and trunk (F). Expression of MRF4-XI line-1 is shown for E17.5 (G, sagittal section) and E18.5 (H). Tongue, proximal limb muscles, some neck and trunk muscles are stained. Abbreviations: bif- biceps femoris; d- deltoid; dg- digastricus; dia- diaphragm; gp- greater pectral; ic- intercostal; lc- longus cervicis; ld- latissimus dorsi; lon- longissimus (cervicis or thoracis); rfe- rectus femoris; sc- semispinalis cervicis; sg- superficial gluteal; sm- semimembranous; sp- splenius capitis; stt- straight thoracis; to- tongue; tr- trapezius; trb- triceps brachii.

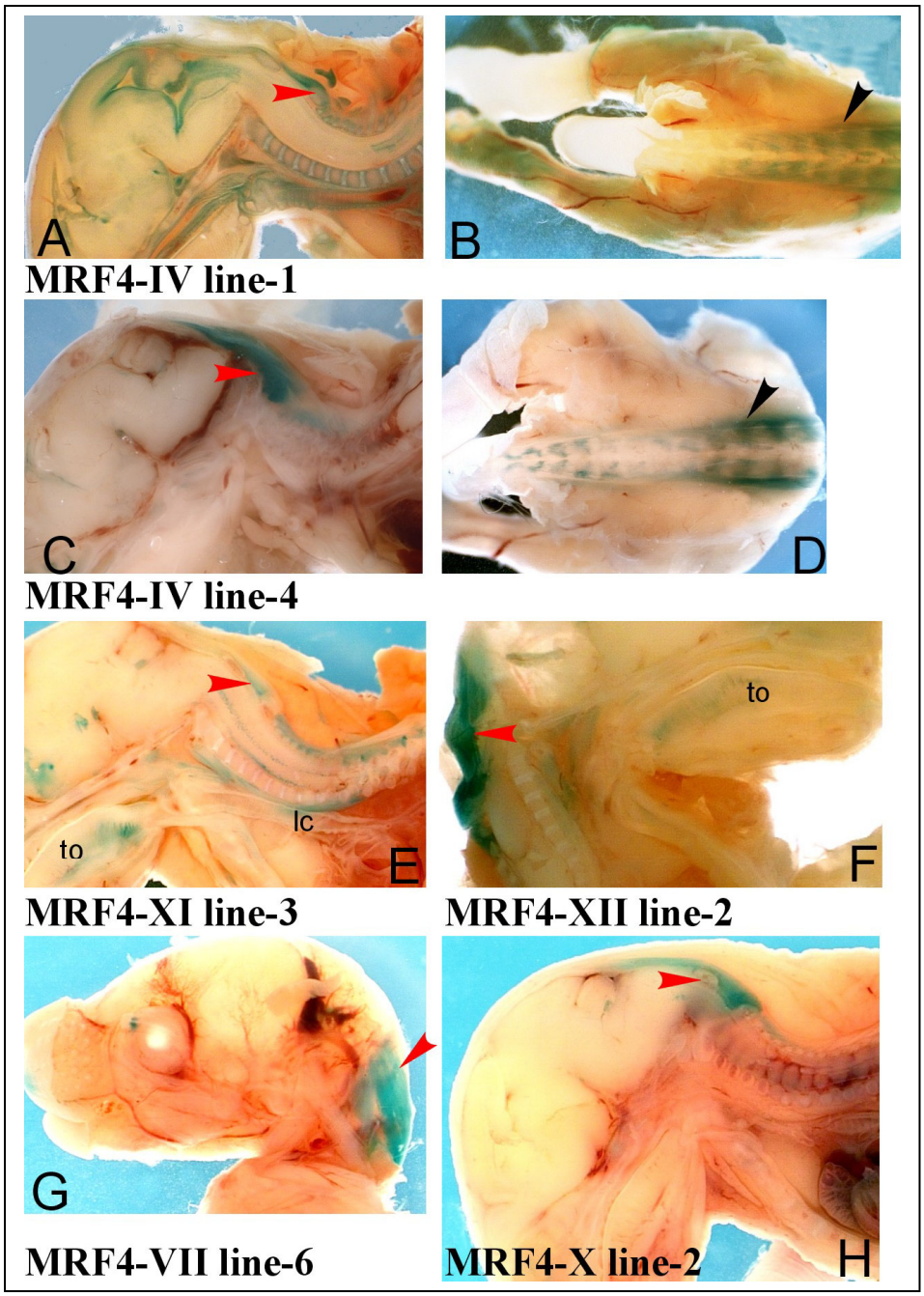

Figure 17. Partial expression pattern of MRF4 transgenes in the neck and lumbar regions. LacZ staining of six mouse lines carrying different transgenes revealed expression in the semispinalis cervicis muscle (red arrowheads in A, C, E-H). For MRF4-IV line-1 (A), MRF4-IV line-4 (C), MRF4-XI line-3 (E), MRF4-XII line-2 (F) and MRF4-X line-2 it is shown on sagittal sections; for MRF4-VII line-6 (G) – on lateral view. Transgenes in the MRF4-IV line-1 (B) and MRF4-IV line-4 (D) are expressed also in lumbar part of paravertebral muscles (black arrowheads). MRF4-XI line-3 (E) and MRF4-XII line-2 (F) show a weak staining in the tongue (to) and MRF4-XI line-3 is expressed in longus cervicis muscle (E, lc). MRF4-IV line-1 shows also the non-muscle ectopic expression in neural tissue and cartilage (A). All embryos are shown at E17.5.

3.3. Interactions between the regulatory elements controlling the MRF4 and Myf5 gene expression and their promoters

3.3.1. The MRF4 promoter responds to the strong -58/-48 kb enhancer when both elements are juxtaposed

An important aspect of MRF4 regulation concerns the fact that the MRF4 and Myf5 genes are closely linked in the same transcriptional orientation but exhibit different expression profiles. It was shown previously that a strong control element situated between -58 kb and -48 kb upstream of the Myf5 gene directs Myf5 expression in limb buds, hypoglossal cord, the myotome, and in brain (Hadchouel et al., 2000). Further analysis revealed a 2 kb enhancer (-57.7/-55.9) within this region (Buchberger et al., 2003). This enhancer contains two sequences, which are highly conserved between mouse and human. One of these homology regions, H1, is required and sufficient to drive Myf5 expression in limbs and maintain it in somites. A second one, H2, seems necessary for Myf5 transcription in occipital somites.

The MRF4 gene has a different, although partially overlapping expression profile. Particularly, MRF4 mRNA was not detected in limb buds until E15.5, and not in hypoglossal cord and in brain. Thus, the MRF4 gene is apparently not regulated by this Myf5 enhancer, despite the fact that the MRF4 promoter lies closer to it than Myf5 (-50/-48 kb). This raises the question of how this promoter selectivity may be established.

One possibility could be that properties inherent to the MRF4 promoter itself may prevent its regulatory interactions with the enhancer. In order to test this hypothesis, the 2-kb Myf5 enhancer was placed directly in front of the MRF4 minimal promoter (the same –3/+3.9 kb fragment, as in the construct MRF4-V) as this promoter fragment alone was incapable of driving expression of the reporter gene in the myotome (Fig. 7 E, F). Together with the 2-kb Myf5 enhancer, however, the MRF4 promoter displayed an expression pattern similar to that of the Myf5 gene (to compare, Fig. 5 A, D in Buchberger et al., 2003). The expression was observed in somites, limbs, and head mesenchyme (Fig. 18 A-G),

indicating that the MRF4 promoter is in principle responsive to the Myf5 enhancer when placed in close vicinity.

The MRF4 promoter- Myf5 enhancer selectivity could also be associated with the proximal MRF4 regulatory elements such as the myotomal control sequence that was characterized in this study. To analyze this possibility, the 2-kb myotomal enhancer was linked directly to the -11.4 kb MRF4 promoter fragment (the same, as in construct MRF4-IV). As with the minimal promoter fragment, even the larger MRF4 upstream sequence could not prevent the enhancer driving expression in limb buds (Fig. 18 H, I). Thus, the MRF4 promoter can in principle interact with the distal Myf5 enhancer and 11.4 kb of MRF4 upstream sequence is not sufficient to spare this promoter from the influence exerted by the enhancer. Therefore, the in vivo pattern of MRF4 expression cannot be explained by selective promoter-enhancer interactions.

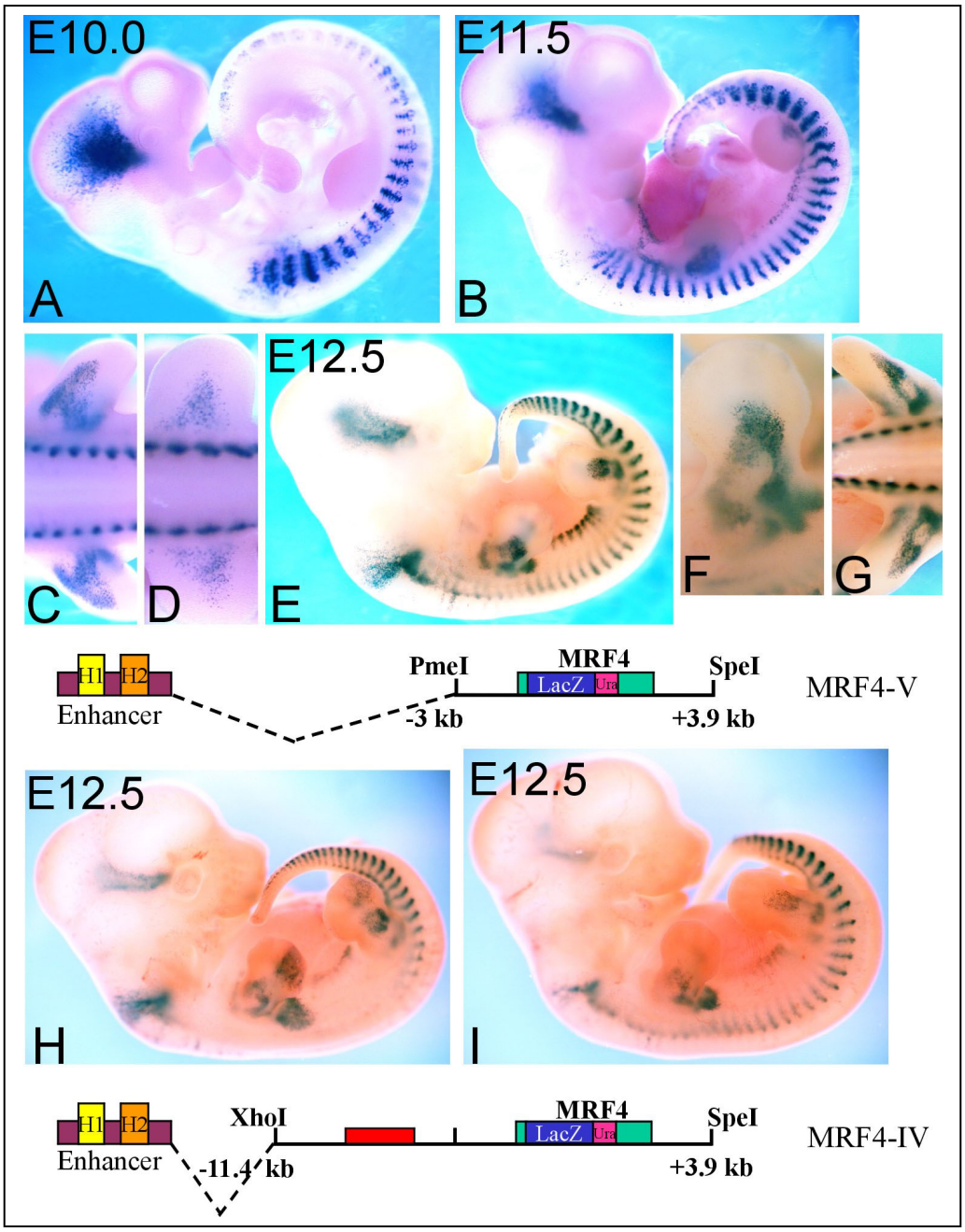

Figure 18. Effect of the Myf5 limb enhancer on MRF4 transgene expression. Panels A-G show expression of the –3 kb promoter plus the 2-kb enhancer in somites and limbs assayed by LacZ staining. Enlarged forelimbs from (B) are shown in (C) and hind limbs in (D), and enlarged forelimbs from (E) are shown in (F) and hind limbs in (G). The longer transgene, which is ectopically expressed in limbs, is shown at E12.5 in (H) and (I). The constructs MRF4-V and MRF4-IV plus the 2-kb enhancer are shown schematically below the panels, respectively. H1 and H2 designate elements with high homology with human genome. The light red box on the lager construct represents the MRF4 myotomal regulatory element (-6.6/-5.6 kb).

3.4. The role of Myf5 protein in the regulation of MRF4 gene expression

Critical transcription factors for embryonic MRF4 expression have not been defined yet. However, genetic evidence argues that Myf5 and MyoD act as determination factors in myogenesis upstream of MRF4 (Arnold & Braun, 2000).

The first Myf5 mutant mouse demonstrated delay of MRF4 expression until E10.5 when MyoD was expressed (Braun et al., 1992). Another Myf5 mutant allele with LacZ insertion into the open reading frame confirmed this delay of MRF4 expression until MyoD was switched on. Moreover, the expression of MyoD itself was also attenuated in this mutant (Tajbakhsh et al., 1996b).

In the present study the role of Myf5 in MRF4 regulation was investigated using a novel Myf5 allele generated by Kaul and colleagues ($Myf5^{\Delta loxP/\Delta loxP}$; Kaul et al., 2000). In this mouse the neo gene flanked by LoxP elements was removed by Cre-mediated recombination. The advantage of using this allele was that these mutant mice were alive and did not have the rib defects described for earlier mutations (Braun et al., 1992; Tajbakhsh et al., 1996b).

3.4.1. Activation of MRF4 expression in dorsal and in ventral domains of myotome occurs independently of Myf5

In order to reveal the role of Myf5 in activation of the MRF4 gene in early myotome a detailed analysis of endogenous MRF4 expression was performed in Myf5 hetero- and homozygous embryos at ss25- ss29.

Two separate expression domains were described previously for the MRF4 gene during early developmental stages (Summerbell et al., 2002). One was initially detected in the middle part of the dorsal half of rostral somites from where expression spreads in dorso-ventral direction. In Myf5 homozygous mutants MRF4 transcripts were not detected initially in the central part of the myotome but appeared more dorsally as soon as the expression spreads in dorsal direction (Fig. 19 A, A1-A4). It is also noteworthy that MRF4 expression in

homozygous Myf5 mutants was not observed in the first three cervical somites (Fig. 19 A, black arrow). In contrast, heterozygous and wild type embryos expressed MRF4 in five cervical somites (Fig. 19 B, Fig. 2 B).

A second expression domain of MRF4 appeared in the ventral part of more caudal somites posterior to the forelimbs. The signal in this ventral domain was quite intense in hetero- and homozygous embryos (Fig. 19 A2-A5, B2-B5).

In summary then, expression of MRF4 in Myf5 mutants seems to initiate correctly in both, the dorsal and in ventral myotome but is missing in the middle part, the so-called intercalated myotome (Fig. 19, red arrows).

3.4.2. Early expression of MyoD in Myf5 mutants

It was suggested previously that MRF4 expression is rescued by the MyoD protein in the Myf5 mutants and that MyoD itself is delayed in these embryos (Braun et al. 1992; Tajbakhsh et al. 1996b). The present analysis of the new Myf5 mutant allele revealed no delay in MRF4 activation. It was, therefore, interesting to examine MyoD expression in this mutant mouse.

The first MyoD transcripts appeared in the ventral part of the four somites just caudal to the forelimbs at ss25. This early expression pattern of MyoD was undistinguishable in hetero- and homozygotes (Fig. 20). Thus, MyoD expression seems not delayed in the Myf5 mutant. Moreover, MyoD expression appeared initially in the same domain where MRF4 was already expressed. Therefore, it seems unlikely that MyoD acts as the MRF4 activation factor in the Myf5 mutants.

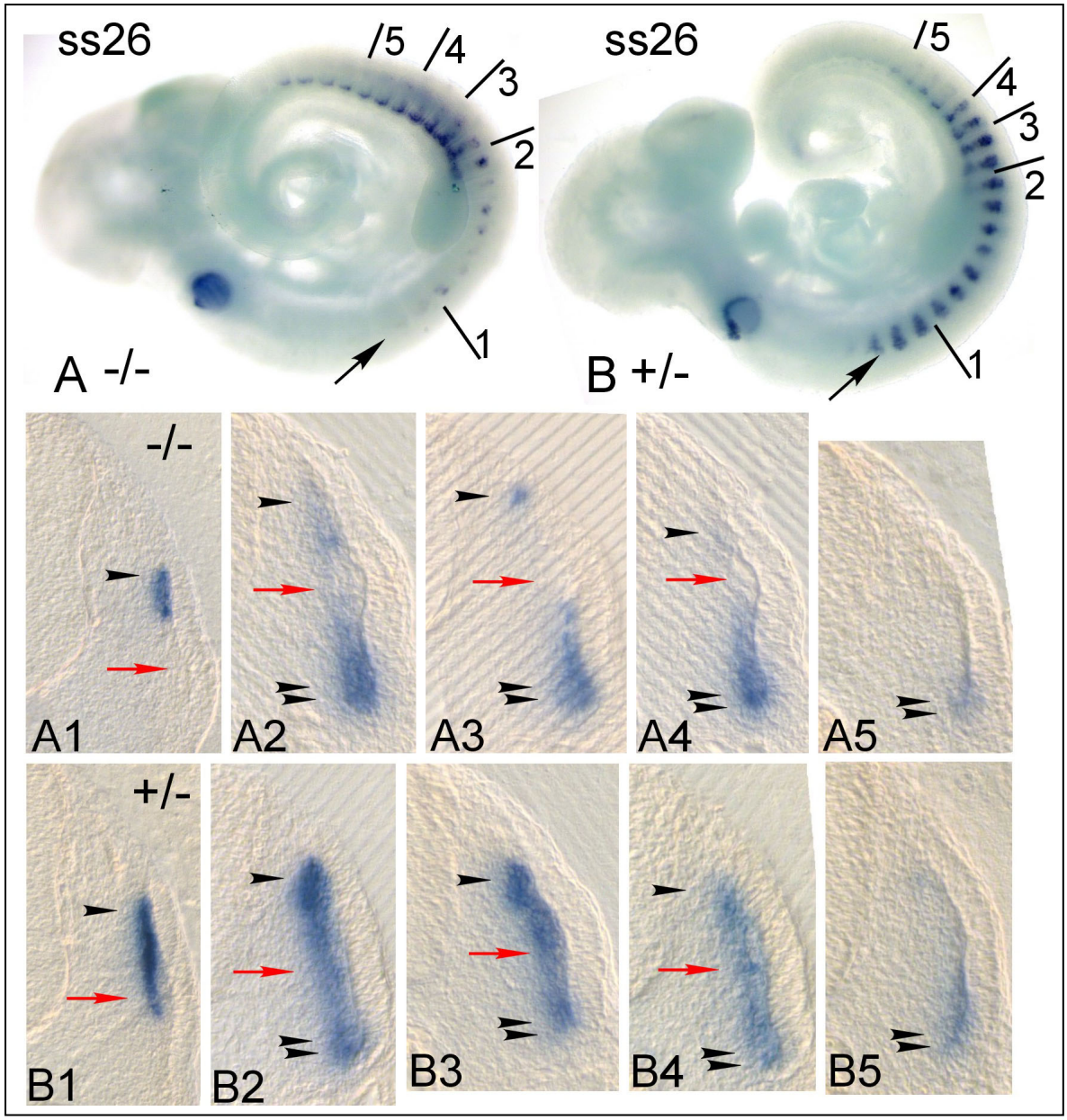

Figure 19. Activation of the MRF4 gene in Myf5 mutants. Myf5-/- (A) and Myf5+/- (B) embryos at ss26 were hybridized with the MRF4 riboprobe and sectioned (A1-5, B1-5). Black lines show planes of sections in the corresponding embryos. Expression is activated independently of Myf5 protein in epaxial (black arrowheads) and hypaxial (black double arrowheads) domains in both homo- and heterozygotes. Expression in the intercalated domain (red arrow) is missing in Myf5-/- embryos. No MRF4 transcripts were detected in the rostral cervical somites of Myf5-/- embryos (black arrow in A, compare with B).

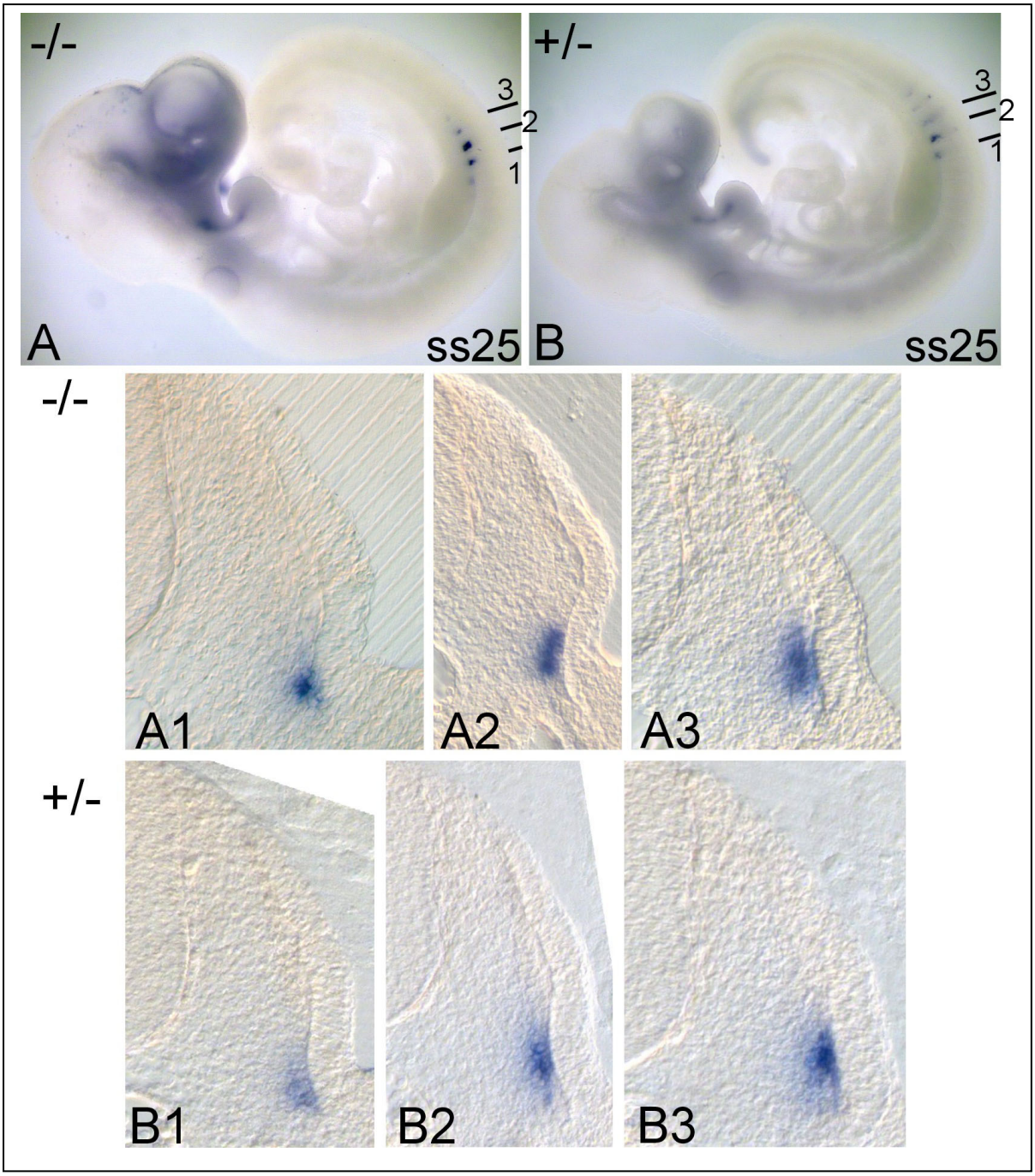

Figure 20. MyoD expression starts at the correct time in Myf5 mutants. In situ hybridization with the MyoD probe of Myf5-/- (A) and Myf5 +/- (B) embryos reveals no difference in expression patterns at ss25. Expression appears in the hypaxial myotome of somites located caudal to the forelimbs (A1-3, B1-3). Black lines in the corresponding embryos show planes of sections.

3.4.3. Myf5 is necessary for myogenesis in the intercalated myotome

MRF4 expression was not detected in the central myotome of Myf5 mutants at E9.5. In order to examine whether this defect might be rescued later during myotome development, in situ hybridization was performed on mutant embryos at E10.5, E11.5 and E13.0.

The results demonstrate that whereas epaxial and hypaxial parts of the myotome had comparable levels of MRF4 expression in hetero- and homozygotes, the intercalated myotome of the homozygotes failed to express MRF4 transcripts at any developmental stage (Fig. 21, red arrowheads).

MyoD expression in Myf5 mutants was also not detected in the intercalated myotome of young and mature somites at least until E13.0 (Fig. 22, red arrowheads). All other aspects of MyoD expression were found similar in the hetero- and homozygotes. Particularly, no differences were observed in branchial arches, limb buds, and epaxial and hypaxial parts of the myotome at E10.5-E13.0 and in forming muscles at E13.0 (Fig. 22).

The defect in expression of myotomal markers was observed in the intercalated myotome of the Myf5 deficient embryos. This raises the question whether the somite structure is affected in these mutants. Two gene markers were used to check the integrity of the intercalated dermomyotome in the Myf5 mutant. Engrailed 1 (En1) is expressed in the intercalated region of the epaxial dermomyotome. The Single minded 1 (Sim1) gene, which encodes a bHLH factor, is expressed in the central dermomyotome region that overlaps the En1 territory yet expands more ventrally (Spörle, 2001).

In situ hybridization was performed on E9.5-E12.5 hetero- and homozygous Myf5 mutant embryos with En1 and Sim1 probes. No difference was detected between mutants and heterozygous embryos at any developmental stage with En1 or Sim1 probe. As it is shown for E10.5 embryos in Fig. 23, the medio-lateral patterning of the dermomyotome was unaffected in the Myf5 mutants and central dermomyotome markers were expressed correctly.

3.4.4. Myf5 is required for MRF4 expression in the most rostral cervical somites

As it is mentioned above, rostral somites of E9.5 Myf5-/- embryos failed to express MRF4 (Fig. 19 A). MRF4 expression was also not restored in the most rostral cervical somites at E10.5. While in heterozygous and wild type embryos the signal was found in four or five cervical somites (Fig. 21 B, C, black arrows), in the mutants MRF4 mRNA was detected only in three cervical somites located just rostral to the forelimbs (Fig. 21 A). On later stages MRF4 expression ceases in the rostro-caudal direction and normally is not detected in cervical somites at E11.5.

In contrast to MRF4 expression, MyoD transcripts were detected in the same number of cervical somites in homozygous and heterozygous embryos at E10.5- E13.0 (Fig. 22). Thus, it implicates that the Myf5 protein is required for MRF4 but not for MyoD expression in the cervical somites.

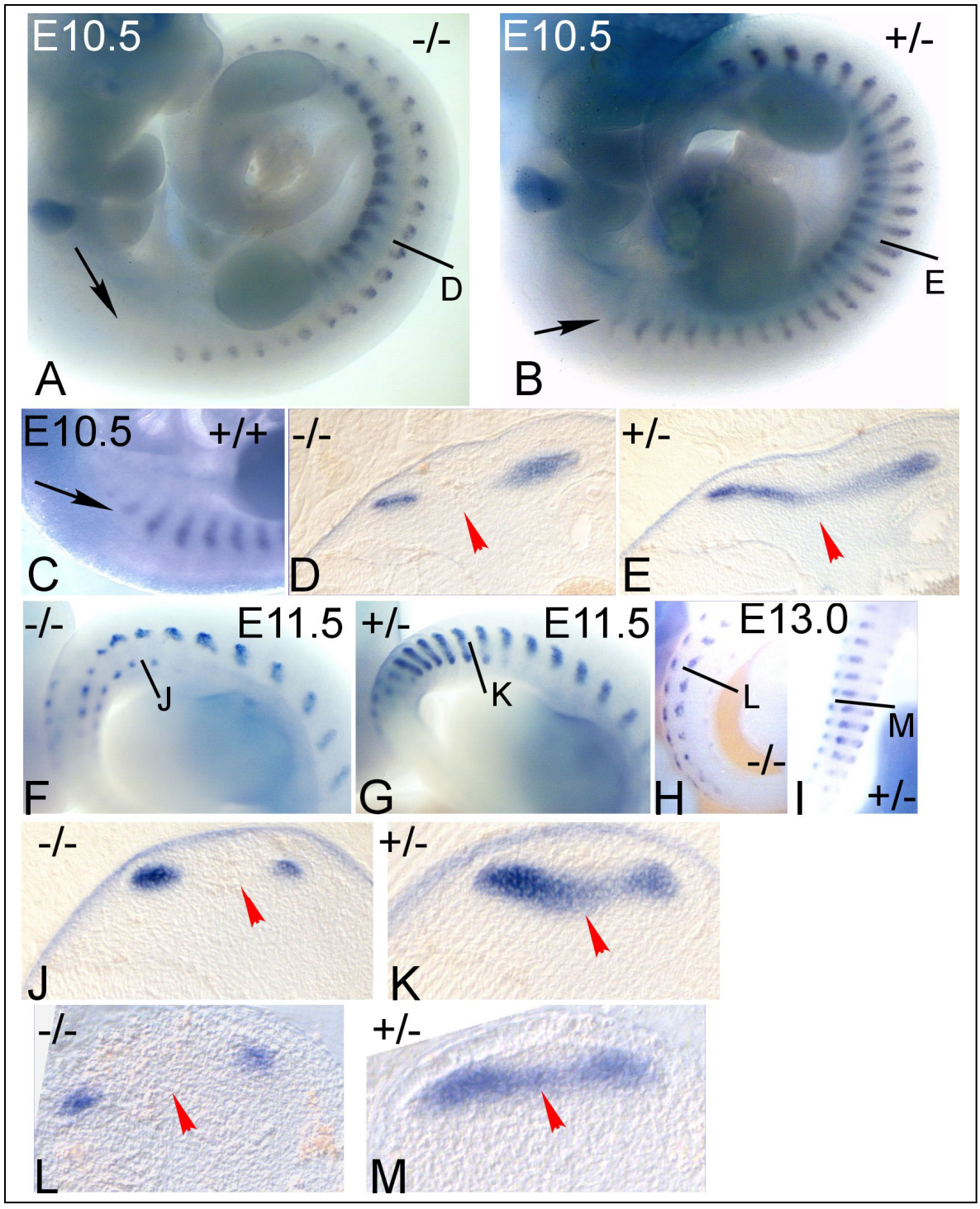

Figure 21. The intercalated myotome of Myf5 mutants fails to express MRF4 until E13.0. Myf5-/- embryos at E10.5 (A, D), E11.5 (F, J), E13.0 (H, L) and Myf5+/- embryos at corresponding stages (E10.5- B, E; E11.5- G, K and E13.0- I, M) are hybridized with the MRF4 probe. Whereas expression in the epaxial and hypaxial domains is unaffected in Myf5 mutants, the signal in the intercalated domain is missing (red arrowheads). Black lines show the planes of sections. Expression in rostral cervical somites is missing in the homozygous embryos (black arrow in A, compare to the heterozygous (B) and wild type embryos (C).

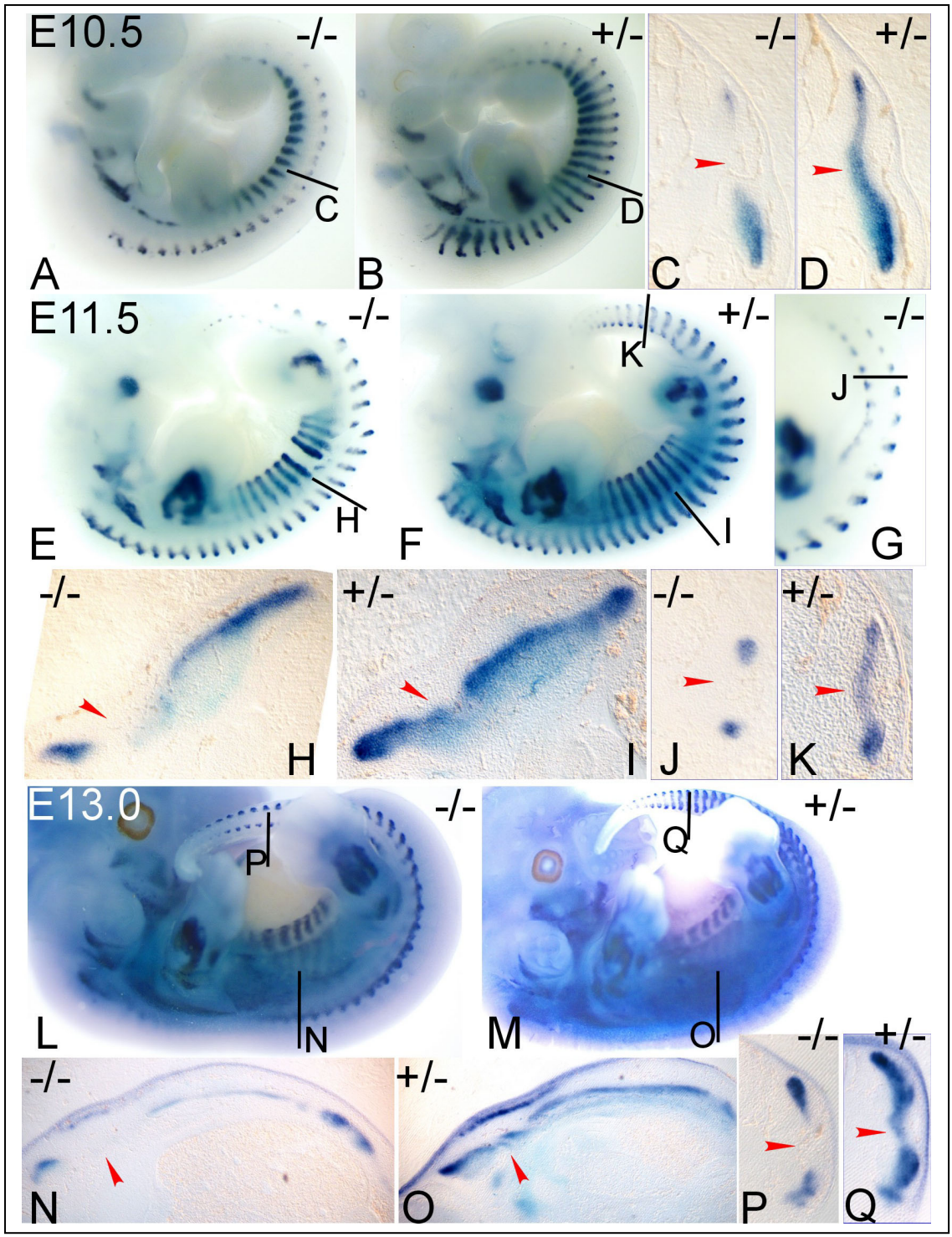

Figure 22. MyoD expression is impaired in the intercalated myotome of Myf5 mutant embryos. Expression at E10.5 (A, C), E11.5 (E, G, H, J) and E13.0 (L, N, P) is missing in the intercalated domain of young and mature somites of Myf5-/- embryos (red arrowheads, compare to the heterozygous littermates: E10.5- B, D; E11.5- F, I, K; E13.0- M, O, Q). Black lines on the corresponding embryos show planes of sections.

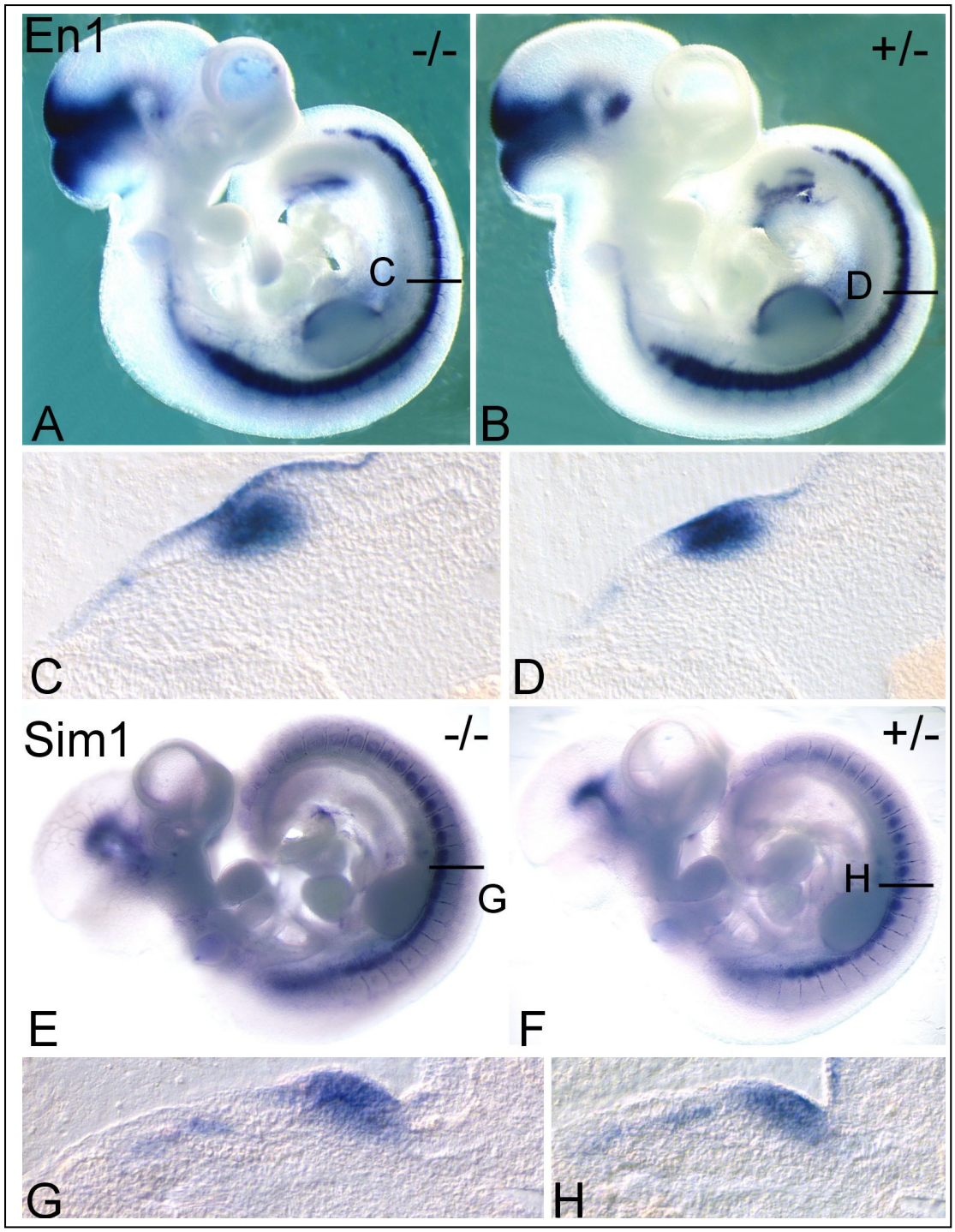

Figure 23. Expression of the central dermomyotome markers En1 and Sim1 is unaffected in the Myf5 mutant. In situ hybridization with the En1 probe (A-D) and the Sim1 probe (E-H) reveals no difference in expression of these markers in the central dermomyotome of Myf5-/- embryos (A, C, E, G) and Myf5+/- embryos (B, D, F, H), at E10.5. Black lines indicate planes of section.

3.4.5. Lack of Myf5 affects the transgenes MRF4-III and MRF4-X similar to the endogenous gene

In order to investigate the effect of Myf5 on MRF4 transgene activity, two stable transgenic lines carrying MRF4-III and MRF4-X, respectively, were crossed into $Myf5^{\Delta loxP/\Delta loxP}$ mice. The expression pattern in MRF4-III line-1 was similar to the endogenous MRF4 gene with the exception of the early ventral domain in caudal somites, which is not driven by this construct.

No expression of MRF4-III was observed in Myf5 homozygous mutant embryos at ss17 (Fig. 24 A), whereas in heterozygous embryos the transgene was detected at ss16 in five cervical somites (Fig. 24 B). In the homozygous embryo at ss22, MRF4-III was activated in three cervical and seven thoracic somites (Fig. 24 C). The transgene appeared in the dorsal but not in the central part of the myotome, and was not expressed in the most rostral somites, similar to the endogenous MRF4 gene in Myf5 mutants. In contrast, the transgene was detected in all cervical somites of heterozygous embryos at ss23 (Fig. 24 D). In Myf5 mutant embryos at E10.5-E12.5, expression of the transgene was very similar to the endogenous MRF4 gene (Fig. 24 E, I, M). β-galactosidase staining was restricted to the epaxial and hypaxial myotome but was missing in the intercalated myotomal region (Fig. 24 F, J, N, red arrowheads). It was also missing in the most rostral cervical somites of the Myf5 mutant at E10.5 and E11.5 (Fig. 24 E, I, black arrows; compare with G and K). It should be mentioned that the MRF4-III construct contained the entire Myf5 gene, potentially leading to some expression of Myf5 protein, which may partially rescue the Myf5 null phenotype. To rule out any effect of Myf5 that may be produced from the transgene, MRF4-X, which did not contain the Myf5 gene sequence, was also tested.

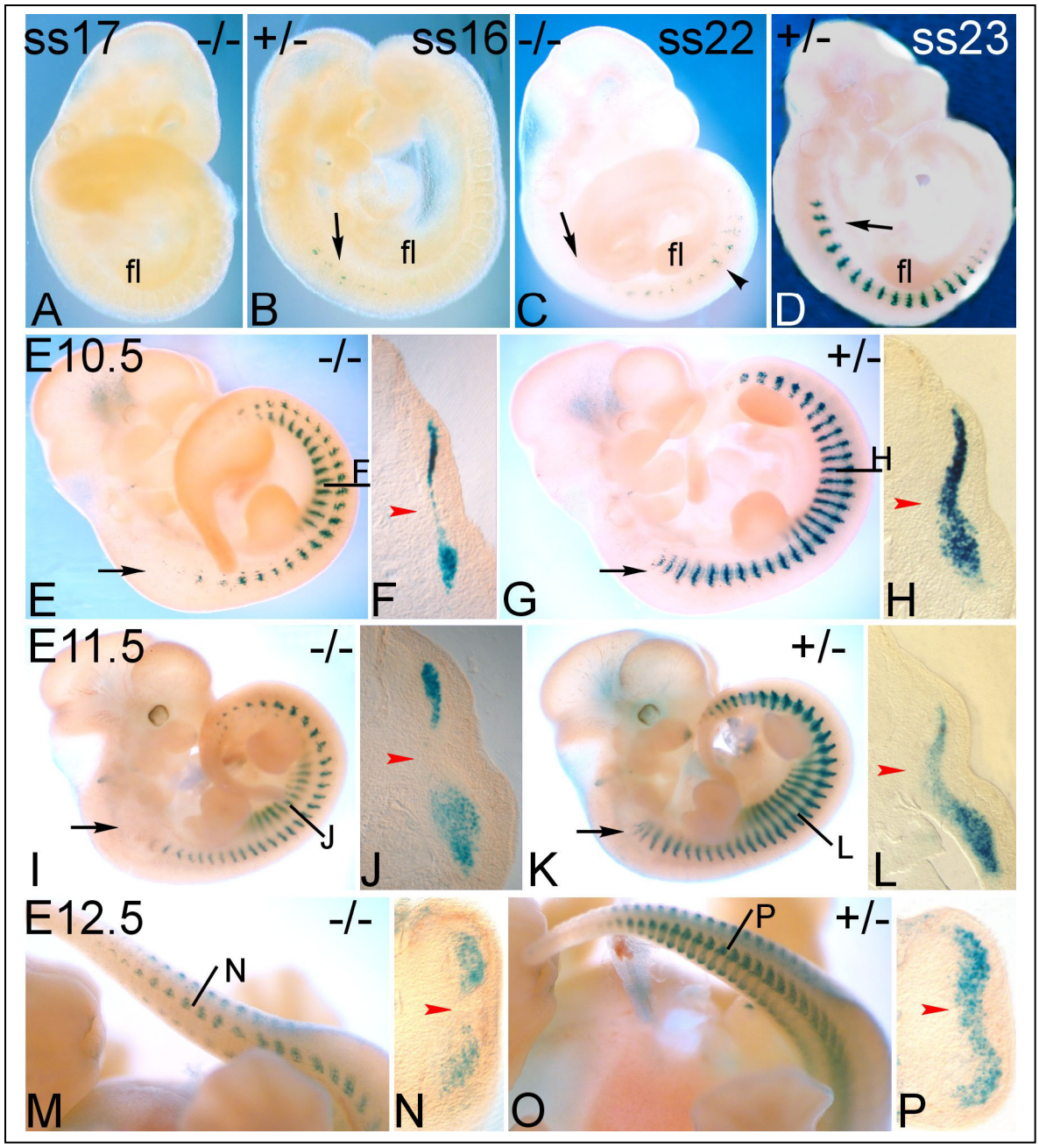

Figure 24. Myf5 is required for MRF4-III transgene expression in cervical somites and in the intercalated myotome. Transgene expression was assayed by LacZ staining of Myf5+/- and Myf5-/- MRF4-III transgenic embryos. Expression of the transgene appears in cervical somites of the heterozygous embryos at ss16 (black arrow in B), but is missed in the Myf5-/- embryos at this stage (A). When expression proceeds in caudal direction and is detected in all cervical somites and most of thoracic somites of Myf5+/- embryos (D), it is found also in 3 somites rostral to the forelimb and 7 thoracic somites of Myf5-/- embryos (black arrowhead in C). At E10.5 and later on, somitic expression is rescued in the epaxial and hypaxial domains but it is still absent in the intercalated myotome of Myf5-/- embryos (E-P, red arrowheads in sections). Expression in 4 rostral cervical somites is missing in homozygous embryos (black arrows; compare A to B, C to D, E to G, I to K). Black lines show planes of section. “fl”, forelimb.

As mentioned before, the MRF4-X construct contains the 1-kb regulatory fragment, which drives MRF4 gene expression in myotomes along the anterior-posterior axis. This region contains several E-boxes, which may serve as Myf5 binding sites. In order to investigate whether the MRF4 regulation by Myf5 can be mediated through the described myotomal control element of the promoter proximal region, the MRF4-X transgenic mouse line 2 was crossed into the Myf5 mutant mouse. Transgene expression was observed in the epaxial myotome of Myf5 mutant at E9.5- E12.5 (Fig. 25 A, E, I) and in the hypaxial myotome of thoracic somites at E11.5 (Fig. 25 I, black arrowhead). The intercalated myotome of the Myf5 mutant failed to express the MRF4-X transgene at any stage like the endogenous MRF4 gene (Fig. 25 B, F, J, red arrowheads). With this transgene it was difficult to assess transgene expression in cervical somites of the Myf5 mutant because of some ectopic expression in the epaxial dermomyotome of the most rostral somites and in adjacent mesenchyme.

In summary, the results of MRF4 transgene expression in the absence of Myf5 argue that Myf5 is required for MRF4 expression in the most rostral somites and in the intercalated myotome of all somites along the anterio-posterior axis. Expression of MRF4 in the epaxial and hypaxial myotomal domains appears independent of Myf5 protein.

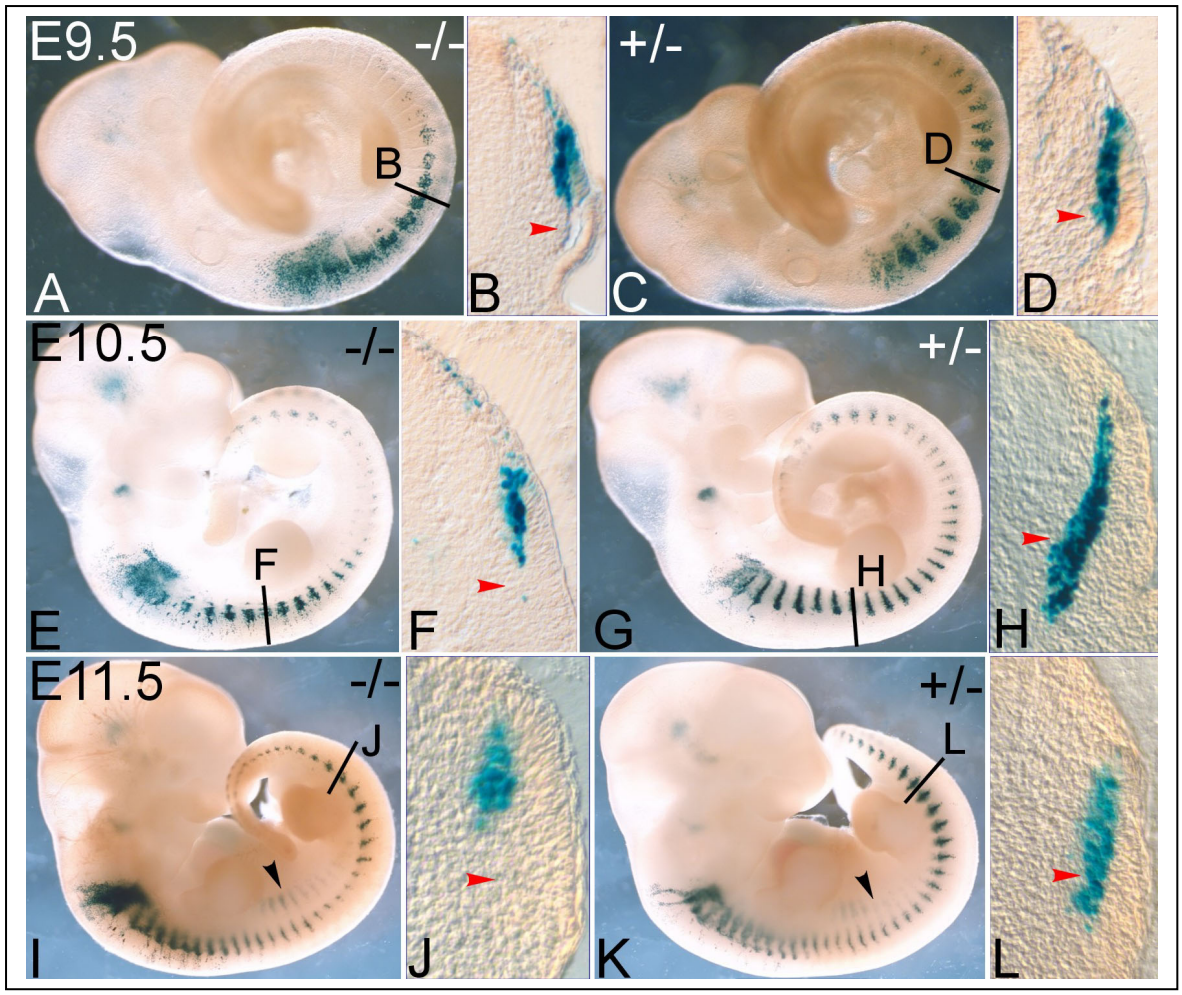

Figure 25. Expression of the MRF4-X transgene in Myf5 mutant embryos assayed by LacZ staining. This transgene is not expressed in the intercalated myotome of Myf5-/- embryos (A, B; E, F; I, J; red arrowheads on the sections, compare to Myf5+/- embryos, C, D; G, H; K, L). Expression in the epaxial region is normal also in Myf5 mutants. Black arrowheads show hypaxial expression in thoracic somites of both homozygous (I) and heterozygous (K) embryos at E11.5. Black lines on the corresponding embryos show planes of section.

4. Discussion

Development of skeletal muscles is coordinated by the myogenic regulatory factors, Myf5, myogenin, MRF4 and MyoD (reviewed in Arnold & Braun, 2000). These transcription factors have different, though partially overlapping expression patterns and play distinct roles in initiation and maintenance of the myogenic program. Studying the mechanisms underlying the control of specific MRF expression in all regions where muscle formation occurs is, therefore, crucial for a better understanding of the complex processes, which govern myogenesis.

The regulation of MyoD and myogenin gene expression has been studied and shown to be dependent on relatively simple elements. In contrast, the control of Myf5 and MRF4 expression is far more complex. Recent studies revealed multiple regions within the Myf5 locus, which are involved in its expression control. A number of distinct elements controlling several aspects of the spatiotemporal pattern of Myf5 expression have been identified (Buchberger et al., 2003; Carvajal et al., 2001; Hadchouel et al., 2000; Summerbell et al., 2000; Zammit et al., 2004).

This study aimed to investigate the regulation of MRF4 gene expression during embryonic myogenesis in the mouse. Of particular interest was the identification of *cis*-acting control elements which direct the biphasic expression of the MRF4 gene. To this end, a series of constructs carrying different length promoter fragments of the MRF4 gene together with the nLacZ reporter gene were used to analyze transgene expression in transient transgenic embryos and in stable transgenic mouse lines. Several regulatory elements were identified in location relatively close to the MRF4 gene (Fig. 26). In addition, it was shown that these control elements are not sufficient to protect the MRF4 promoter from non-selective interactions with enhancers, which are dispersed throughout the MRF4/Myf5 locus. Finally, the importance of Myf5 protein for controlling MRF4 expression was investigated in Myf5 deficient mutant mice.

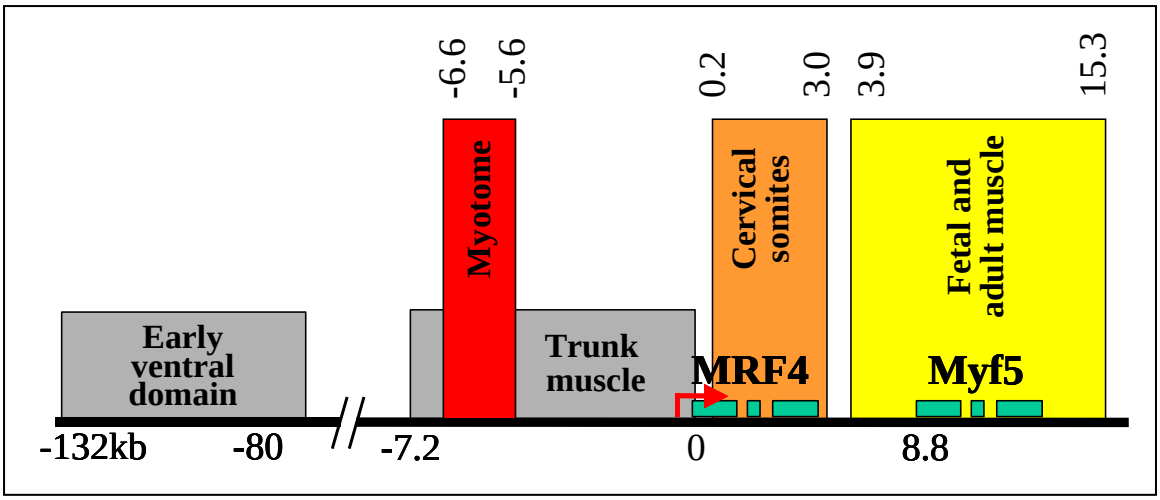

Figure 26. Regulatory regions within the MRF4 gene locus. Previously characterized domains* are shown as gray boxes and novel regions identified in this study as color bars. Numbers indicate the location relative to the transcription start of the MRF4 gene. Green boxes indicate exons of the MRF4 and Myf5 genes. Red arrow indicates direction of transcription. The map is not drawn to scale.

*Carvajal et al., 2002; Patapoutian et al., 1993.

4.1. Elements controlling MRF4 expression in the myotome

The elements controlling the embryonic phase of MRF4 expression had not been defined in any detail. Somitic expression along the entire body axis was obtained with a mouse BAC clone containing 50 kb upstream and 48 kb downstream sequence of the MRF4 gene (Carvajal et al., 2001), while a 7.2-kb MRF4 promoter fragment did not support myotomal expression (Patapoutian et al., 1993). Taken together these data argued that the regulatory control elements are located between –50 and –7.2 kb or (and) between +64 bp and +48 kb. Pin et al. obtained expression in the central myotome of thoracic but not of rostral and caudal somites using an 8.5-kb fragment of the MRF4 rat promoter (Pin et al., 1997).

The present analysis of the MRF4 gene in transgenic animals provides first evidence that regulation of most aspects of the embryonic expression in myotomes of all somites resides in a region located relatively close to the MRF4 promoter. A major difference between the patterns obtained with transgenes used in this study and the endogenous MRF4 gene concerns the lack of the early ventral myotomal domain and variability of transgene expression in the hypaxial myotome at later stages (E10.5- E12.5). None of the transgenes used here, showed expression in the ventral domain at E9.0- E9.5, suggesting that additional control elements are necessary for this MRF4 expression domain. Indeed, it has been found previously that a regulatory element, situated in the –132/-80 kb region, is required for MRF4 expression in the early ventral myotome and later in the hypaxial region (Carvajal et al., 2002). It is important to note, however, that expression in the later hypaxial myotome was frequently observed with stable transgenic mouse lines, which initially lacked the early ventral domain in the myotome. It seems, therefore, that expression in early ventral and in late hypaxial domains may be controlled by different elements and mechanisms.

The detailed analysis of various parts of the 7.5-kb sequence upstream of the MRF4 gene revealed a regulatory element located between –6.6 and –5.6 kb which mediates MRF4 expression in the myotome, when combined with the –3 to

+3.9 kb MRF4 gene fragment (MRF4-X). The spatiotemporal pattern of this transgene is very reminiscent of the endogenous MRF4 gene, except for the hypaxial domain described above. The transgenes MRF4-V and MRF4-VIII in which the –6.6/-5.6 kb fragment was removed were essentially not expressed in the mouse embryo, although they contained the minimal promoter. In contrast, this minimal promoter fragment used in transfection experiments of cultured myogenic cells clearly promoted expression (Black et al., 1995). In vivo, however, neither the –3/+3.9 kb sequence nor –5.6/+2.4 kb sequence alone were sufficient to direct transgene expression in somites.

It is yet unknown whether the –6.6/-5.6 kb MRF4 element acts as a classical enhancer that also interacts with heterologous promoters or whether it cooperates exclusively with the MRF4 promoter. The homologous genomic context in this study was intentionally maintained in order to avoid the potentially artificial influence of a heterologous promoter. It has been shown previously that the early epaxial Myf5 enhancer regulates heterologous promoters quite differently from the corresponding endogenous Myf5 promoter (Teboul et al., 2003; Gustafsson et al., 2002). This enhancer when juxtaposed to the viral thymidine kinase promoter serves as a direct target for Shh signaling mediated by Gli transcription factors (Gustafsson et al., 2002), while linked to the Myf5 promoter this enhancer neither depends on Shh function nor on intact Gli binding site for early epaxial Myf5 expression (Teboul et al., 2003).

Previous work by Patapoutian et al. (1993) used the 7.2 kb sequence (BamHI/SalI fragment) upstream of the MRF4 gene to drive expression of the LacZ reporter in mice. These authors did not observe expression during early somite development, although the examined MRF4 sequence actually overlaps with the gene region studied here including the regulatory element at –6.6/-5.6 kb. In contrast to the previous study, most of the constructs used here contain the complete MRF4 gene up to +3.9 kb, which may contribute to efficient and consistent expression in somites. Clearly, however, it is not absolutely required, as the MRF4-VI transgene, which lacks most of the MRF4 gene body and downstream sequence, similar to Patapoutian’s construct, was expressed in our studies. Another difference of Patapoutian’s construct compared to ours was an additional 169 bp of the first MRF4 exon, which also may contribute to stable expression of the gene.

Recently, a new enhancer was mapped at –8 kb upstream of the MRF4 promoter (Chang et al., 2004). This enhancer was isolated by an enhancer trap assay and was able to increase expression of a reporter gene directed by the Myf5 or MRF4 minimal promoter in C2 muscle cells. Interestingly, when fused with the MRF4 and Myf5 promoter transgenes in the mouse were expressed in a mutually exclusive mode. Linked to the MRF4 minimal promoter, the enhancer drove expression either in the embryonic myotome (two stable lines) or in fetal muscles (one line). When it was linked to the Myf5 minimal promoter, only expression in fetal muscle was observed. Therefore, the authors concluded that the enhancer-promoter combination was not neutral and the nature of the promoter, MRF4 or Myf5, seemed to influence the expression pattern of the enhancer. In the present study, deletion of the sequence containing this putative enhancer did not affect the different expression patterns of MRF4 transgenes. Thus, it seems that at least in a more complete genomic context this enhancer is functionally dispensable for the regulation of MRF4 expression in the myotome.

The regulatory region of the mouse MRF4 gene overlaps with that of the previously analyzed rat MRF4 sequence. As shown by Pin et al., the rat transgene was expressed only in thoracic somites (Pin et al., 1997). None of the mouse transgenes examined here elicited an expression pattern limited to thoracic somites only. They were either not expressed in any somites (MRF4-VIII), or were restricted to cervical and tail somites (MRF4-IX), lacking the thoracic region. Despite high sequence conservation of at least 8 kb upstream of both, the mouse and rat MRF4 genes, subtle variations in sequence may be the reason for the observed difference in MRF4 gene regulation between both species.

Computer-based analysis of the 1-kb region which harbors the myotomal regulator of MRF4 expression predicts multiple potential binding sites for transcription factors, such as E-box consensus sequences, binding sites for Mef2 proteins containing the MADS box and cooperating with MRFs, a binding site for TCF/LEF-1, which transduces Wnt signals, the SMAD interacting protein, multiple sites for homeodomain containing factors including Xvent-2 (early BMP signaling response in Xenopus), and members of the Pax family (Fig. 27). These transcription factors or signaling pathways have been implicated in regulation of myogenesis (reviewed in Borycki & Emerson, 2000). Mutation analysis of the myogenin promoter demonstrated the importance of an E-box and a MEF2

binding site for correct spatiotemporal expression of myogenin, indicating that there is a bHLH protein immediately upstream of myogenin in the regulatory cascade (Yee & Rigby, 1993; Cheng et al., 1993; Buchberger et al., 1994). Pax3 is required for MyoD activation and for migration of muscle progenitor cells (Tajbakhsh et al., 1997). Different members of the Wnt family are required for activation of Myf5 and MyoD in vivo and in vitro (Linker et al., 2003; Ikea & Takada, 1998; Tajbakhsh et al., 1998a). Wnt1 was shown to activate Myf5 expression in explants of mouse presomitic mesoderm, whereas Wnt7a differentially activates MyoD expression (Tajbakhsh et al., 1998a). Wnt5b is required for the initiation of MyoD in explants of chick presomitic mesoderm (Linker et al., 2003). Genetic studies of Wnt1/Wnt3a double-mutant mouse embryos revealed reduced level of Myf5 expression at E9.5 (Ikea & Takada, 1998). Bmp signaling plays an inhibitory role in myogenesis preventing premature expression of MyoD before somites form (Linker et al., 2003). However, the role of all these potential binding sites within the MRF4 myotomal control element needs to be tested functionally.

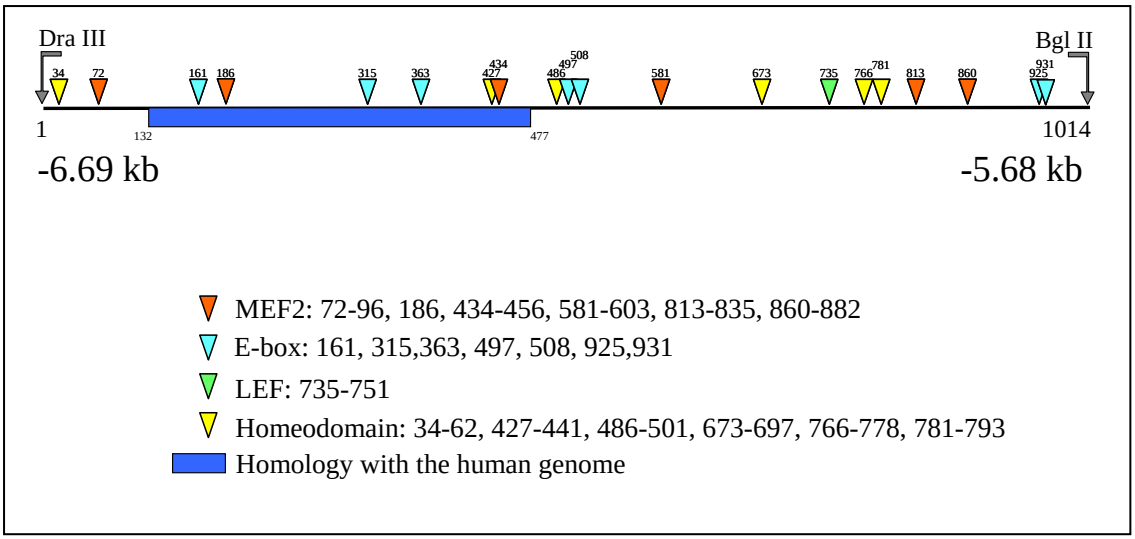

Figure 27. Topological map of potential binding sites for transcription factors within the region essential for early myotomal expression of MRF4 (-5.6/-6.6 kb). Red triangles represent binding sites for the MEF2 transcription factor. Blue triangles – E-boxes; green triangle – the site for the LEF transcription factor; yellow triangles – homeodomain binding sites. Blue box represents the region of high homology with human genome. Numbers designate the position of *cis*-elements on the map relative to the DraIII site.

Sequence comparisons of the 1-kb myotomal mouse element revealed a sequence at –6.5/-6.2 kb with significant homology to the corresponding regions of the human, dog, and chicken MRF4 loci. Conservation of non-coding sequences through evolution has been interpreted as indication for a role in gene regulation. In fact, a conserved element in mouse and Fugu was demonstrated to be critical for Hoxb4 gene expression (Summerbell & Rigby, 2000). Comparative analysis of the mouse genome and the human genome led to the identification of H1 and H2 core elements in the Myf5 limb enhancer (Buchberger et al., 2003). Thus, the conserved sequence within the 1-kb myotomal regulatory element among different species supports its functional significance.

Deletion of 2.8-kb of the MRF4 gene body and sequence downstream of it resulted in loss of transgene expression in occipital and the most rostral cervical somites. This is illustrated by the transgenes MRF4-VI and MRF4-XIV, which revealed expression in tail, trunk, and in the three cervical somites close to the forelimbs but not in more rostral somites. From these results it is suggested that in addition to the upstream element sequence within or downstream of the gene may contribute to expression in anterior somites, although this region alone (the MRF4-V) cannot drive expression in the myotome. Both elements may cooperate to establish the complete MRF4 pattern in rostral somites. Such complexity in gene regulation along the anterio-posterior axis has also been described for the Myf5 gene. An element that activates and maintains Myf5 expression in occipital and cervical somites is located around –57 kb upstream of the Myf5 transcription start site (Buchberger et al., 2003). Additionally, mutation in the Gli-binding site of the Myf5 early epaxial enhancer abolished its expression in maturing cervical and occipital somites (Teboul et al, 2003).

4.2. MRF4 expression in fetal muscles requires multiple control elements

Unlike other myogenic regulatory factors, MRF4 has a biphasic expression pattern. The first wave in myotomes is only transient as expression ceases after E12.5. Starting from E14.5 expression strongly increases in all

skeletal muscles and MRF4 becomes the predominant MRF expressed in late fetal and adult muscle of the mouse (Bober et al., 1991). During this second period of MRF4 expression the protein might be involved in differentiation of myocytes (Rawls et al., 1998; Patapoutian et al., 1995) and possibly later in regeneration processes (Weis et al., 2000).

Previous studies provided evidence that the regulation of MRF4 in the myotome and in fetal muscles is regulated by different elements (Pin & Konieczny, 2002; Patapoutian et al., 1993). The second phase of MRF4 expression in fetal and adult muscles is at least partially regulated by elements within –8.5 kb, as it was shown for the rat MRF4 gene (Pin et al., 1997). The proximal rat MRF4 promoter (-336 bp) exhibits very limited activity in less than 1% of fetal fibers (Pin et al., 1997). An enhancer specific for fast fibers is localized within the -4/-5 kb region (Pin & Konieczny, 2002). The mouse –7.2 kb sequence includes elements, which play a role in MRF4 expression in fetal trunk muscles (Patapoutian et al., 1993).

Some transgenic lines used here would confirm the observation that elements located proximal to the MRF4 promoter are capable to direct expression in fetal and adult skeletal muscles. However, the regulation of expression in fetal muscles appears to be more complex than previously thought. The present study revealed an unexpected role of downstream sequence in the MRF4 gene regulation. Whereas the transgenes MRF4-I, MRF4-II, and MRF4-III containing the +3.9/+15.3 kb genomic sequence downstream of the gene were expressed in all fetal muscles the transgenes in which this fragment was deleted showed no or inconsistent activity during fetal stages. Of more than twenty stable lines and about ten transient embryos with various transgenes lacking the +3.9/+15.3 kb fragment only three stable lines exhibited expression in fetal trunk muscles and six lines were expressed in a single muscle, the semispinalis cervicis muscle in the neck. The expression in individual muscles could be due to position effects at the site of transgene insertion. However, the observed lack of expression in most lines seems unlikely to be the result of silencing by integration effects, since the same lines showed mostly correct expression in myotomes. A plausible explanation for the inconsistent expression in fetal muscles may be that the +3.9/+15.3 kb region may help to stabilize the expression pattern. In previous studies transgenes that did not contain this sequence also exerted variability in

fetal expression (Patapoutian et al., 1993; Pin et al., 1997). For example, only three out of seven transgenic lines containing the –7.5 kb fragment of the mouse sequence (Patapoutian et al., 1993) and three out of six transgenic lines with the –8.5 kb of the rat sequence (Pin et al., 1997) expressed the transgenes in a muscle-specific manner. It is also noteworthy that the –5/-4 kb rat enhancer was active in fast fibers of all muscles in adult animals, whereas the large and overlapping –7.2 kb mouse flanking region drove expression only in the subset of trunk muscles (Pin & Konieczny, 2002; Patapoutian et al., 1993). Thus, additional sequences are required to control MRF4 expression in mouse fetal muscles.

4.3. Models for the promoter-enhancer interactions in the Myf5/MRF4 locus

The close physical linkage of the Myf5 and MRF4 genes, presumably the result of a gene duplication, presents an interesting problem in organization, utilization and evolution of their corresponding regulatory elements. Multiple regulatory elements are dispersed throughout the MRF4/Myf5 locus, some of them at large distances from the MRF4 and Myf5 promoters. In addition, the protein tyrosine phosphatase gene, Ptprq, overlaps with the MRF4/Myf5 locus but is not expressed in skeletal muscles (Wright et al., 1998). Obviously, this gene is regulated differently. The interdigitated arrangement of modular control sequences within the locus raises the question whether, and if so, to what extent the regulatory elements affect the promoters of both myogenic factor genes. The enhancers controlling Myf5 expression in the early epaxial dermomyotome and in branchial arches are localized in the MRF4-Myf5 intergenic region (Summerbell et al., 2000). Since MRF4 is not expressed in the early epaxial dermomyotome and in branchial arches, these enhancers apparently regulate only Myf5 expression and have no influence on the MRF4 promoter. Some of the transgenes examined in this study provide evidence supporting this hypothesis. The transgenes MRF4-I, -II and -III contain the branchial arch enhancer but do not reveal expression in this domain. A mechanism underlying the selective promoter-enhancer interaction in this case may follow the model of promoter competition as suggested for the imprinted Igf2/H19 locus (Webber et al., 1998). This model was also used to demonstrate that there is competition between the

chicken embryonic and adult β-globin genes, which achieve their stage-specific transcriptional regulation through the competition for a single intergenic enhancer (Choi & Engel, 1988). Based on this model the exclusive expression of one of the genes could be explained by either its closer proximity to the enhancers or the inherently greater strength of its promoter. However, this model cannot explain the selective interactions between the early epaxial enhancer (EEE) and the Myf5 promoter. Most of the transgenes used in this study are truncated just downstream of the EEE. As they do not include the Myf5 promoter, promoter competition for the EEE cannot be considered. Even in the context of the relatively short promoter region, –3/+3.9 kb (MRF4-V), expression of the transgene does not follow the Myf5 pattern in the early epaxial dermomyotome. Moreover, results of the Myf5 mutant mice analysis performed in this study also contradict the competition model. In these mutants gene deletion includes the Myf5 promoter (-2/+1 kb) while preserving EEE (Kaul et al., 2000). This mutation, however, does not result in the shift of MRF4 expression toward the Myf5 pattern. The EEE was shown to be active with the heterologous β-globin promoter when placed upstream of it (Summerbell et al., 2000). In the transgenes tested in the present study the enhancer is located downstream of the MRF4 promoter but does not activate it. Therefore, it may be suggested that the position of the enhancer relative to the promoter is critical for its function. Alternatively, a gene insulator or chromatin boundary element could prevent the interaction of the MRF4 promoter and the Myf5 early epaxial enhancer.

The –58/-48 kb regulatory region has been demonstrated to be important for Myf5 expression in limb buds and for certain aspects of the patterns in somites (Buchberger et al., 2003) but it apparently does not activate the MRF4 promoter, at least not in limb buds. This enhancer is located closer to the MRF4 than to the Myf5 promoter excluding, therefore, a model of linear promoter scanning as employed in the HoxD cluster, where the enhancer interacts preferentially with the nearest promoter (Kmita et al., 2002). The results of the present study rule out the possibility of target specificity brought about either by the MRF4 promoter itself or by the nearby regulatory element. The data indicate that the –58/-48 kb enhancer can interact with the MRF4 promoter and the promoter proximal element driving myotomal expression of MRF4 is unable to

prevent this interaction. Thus, a mechanism of selective promoter-enhancer interaction based on target specificity seems very unlikely. Also the competition model discussed above cannot apply in this case, since, as it was pointed out before, the Myf5 promoter sequence was deleted in the Myf5 mutants. However, MRF4 expression was restricted to the myotome and never detected in limb buds. A possible explanation why in vivo the MRF4 promoter is not responsive to the limb specific Myf5 enhancer may be due to the presence of an insulator element (or silencer) located between the -58/-48 kb enhancer and -11.4 kb upstream of the MRF4 gene. The function of this putative element would be to shield the MRF4 promoter from the strong enhancer.

A looping model can also explain the promoter-enhancer selectivity. This model states that enhancers and promoters communicate through direct interactions between proteins bound to them, with the intervening DNA looping out. One example of this model is the mammalian β-globin cluster, where the locus control region (LCR), located 40-60 kb away from the genes, comes in close proximity to the active globin gene looping out the inactive globin gene (Carter et al., 2002; Tolhuis et al., 2002). That looping occurs only during transcription and the same locus adopts an essentially linear conformation in nuclei of nonexpressing cells (Tolhuis et al., 2002). In the case of the MRF4/Myf5 locus, nuclear factors would bind to DNA elements and bring the -58/-48 kb enhancer into close proximity of the Myf5 promoter in the Myf5 expressing cells, whereas the MRF4 gene would be looped out (Fig. 28, B).

Another mechanism underlying the specific interactions within the locus might be analogous to the one described for the Drosophila homeotic gene Abd-B (Zhou & Levine, 1999; Müller, 2000; Lin et al., 2003). The suggested mechanism here is based on an element with anti-insulator activity, the Promoter Targeting Sequence (PTS), which selectively permits some interactions across insulators. In the case of MRF4/Myf5 locus it would mean the presence of an insulator that inhibits the interactions of the enhancer with both MRF4 and Myf5 promoters, and an anti-insulator that prevents insulating of the enhancer for the Myf5 promoter selectively (Fig. 28, C).

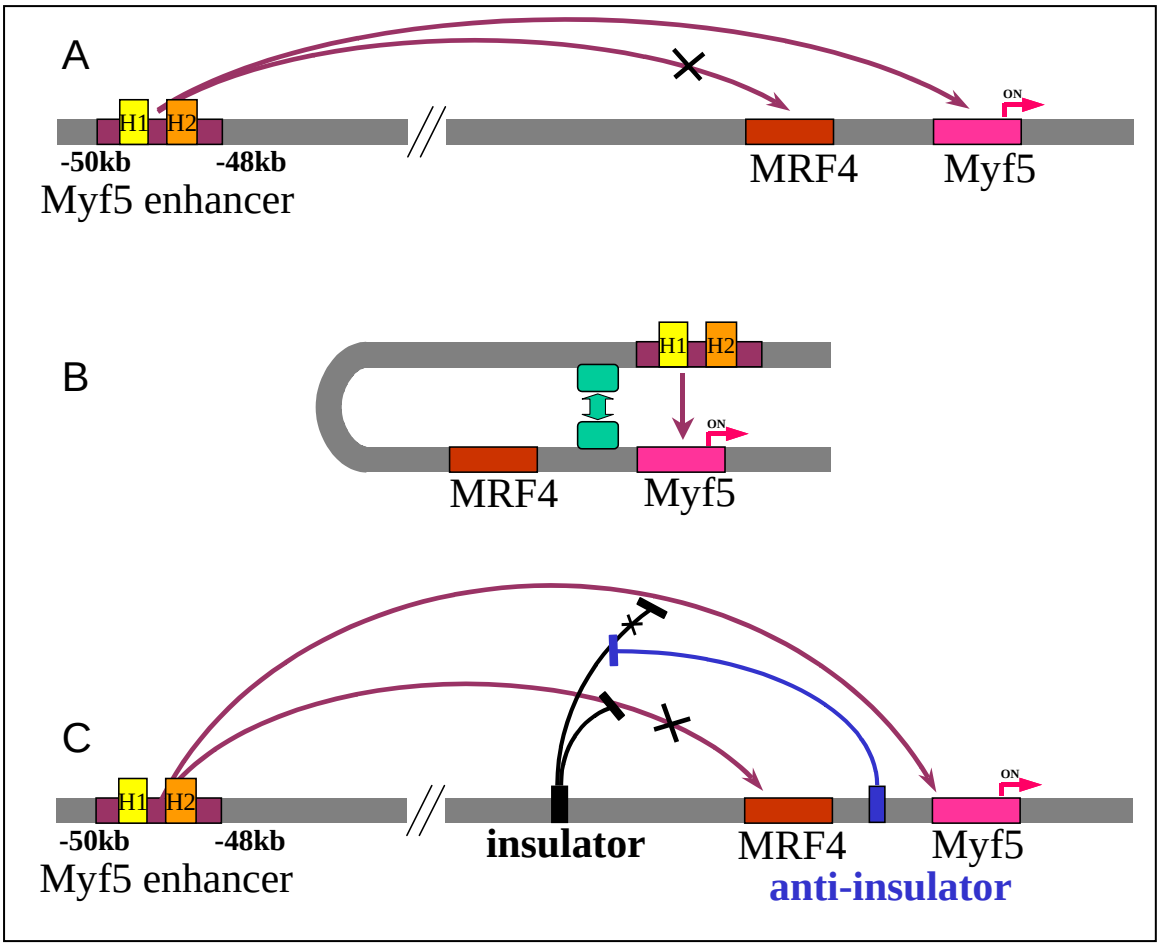

Figure 28. Models of selective promoter-enhancer interaction in the MRF4/Myf5 locus. A: Myf5 limb enhancer located 50 kb upstream of the MRF4 gene selectively activates the Myf5 promoter, but is unable to induce the MRF4 promoter in vivo. B: A looping model predicts specific protein-protein interactions between multiprotein complexes (green rectangles), which would bind to DNA elements and bring the Myf5 enhancer to close proximity with the Myf5 promoter. As a result, the MRF4 gene located between the enhancer and the Myf5 promoter is looping out and, thereby, it avoids the effect of the enhancer. C: Anti-insulator model envisages of the chromatin boundary element between the enhancer and the MRF4 gene, which should block any enhancer downstream activity. The Myf5 gene, however, may overcome the insulator negative effect and respond to the enhancer because of the function of the anti-insulator element located upstream of the gene.

4.4. The role of Myf5 protein in MRF4 gene regulation

The introduction of null mutations of Myf5 and MyoD into germline of mice has revealed the hierarchical relationship among MRFs. Thus, Myf5 and MyoD were shown to be required for cell fate determination of muscle progenitor cells and were proposed to act upstream of MRF4. Myotome development of embryos lacking Myf5 was delayed until the onset of MyoD expression, which occurs with delayed kinetics (Braun et al., 1992; Tajbakhsh et al., 1997). Particularly, MRF4 expression was not detected in Myf5 mutants before MyoD was activated. Newborn mice deficient in both Myf5 and MyoD were totally devoid of myoblasts and myofibers (Rudnicki et al., 1993). However, the proximity of MRF4 and Myf5 to each other on the same chromosome raises the possibility of *cis*-regulation in the locus (Olson et al., 1996). It was demonstrated previously that MRF4 mutations affect Myf5 expression in *cis* (Floss et al., 1996; Yoon et al., 1997). A *cis*-regulatory effect in Myf5 mutants on MRF4 expression also seems possible (Yoon et al., 1997). All analyzed Myf5 alleles containing the neomycin or LacZ and neomycin expression cassettes apparently influence MRF4 expression in *cis*. Therefore, in the present study the $Myf5^{\Delta loxP/\Delta loxP}$ allele was used to investigate the role of Myf5 protein in the regulation of MRF4 expression. In this allele the neomycin gene cassette was removed in the germ line by Cre-mediated recombination of loxP sites flanking the selection gene (Kaul et al., 2000).

In this allele in which MRF4 expression is presumably not compromised in *cis*, activation of the MRF4 gene occurs independent of Myf5 protein. Furthermore, MRF4 transcripts appear on time in the dorsal and ventral domains of the myotome, and before MyoD is expressed. Also, MyoD expression does not seem delayed in $Myf5^{\Delta loxP/\Delta loxP}$ mutants, in contrast to the previously studied $Myf5^{nLacZ/nLacZ}$ embryos (Tajbakhsh et al., 1997). MyoD expression is first detected at ss25 in domains coinciding with MRF4 expression. This would be consistent with the idea that MRF4 promotes MyoD activation in Myf5 mutants. Tajbakhsh and colleagues have recently shown in allelic series of Myf5 mutants that indeed mutations in the Myf5 gene locus can alter expression of MRF4 in *cis* and also concluded that MRF4 might activate MyoD in the absence of Myf5

(Kassar-Duchossoy et al., 2004). Moreover, this group also demonstrated that a subset of skeletal muscles is formed in the Myf5:MyoD double mutants when MRF4 expression is not compromised. Taken together the present analysis of the $Myf5^{\Delta loxP/\Delta loxP}$ allele and results of the Tajbakhsh group indicate that MRF4 expression is activated independently and it is one of the earliest myogenic factors operating in the myotome. It may act in concert with Myf5 and upstream of MyoD to determine myogenic cell fate.

The role of MRF4 as a determination factor was first suggested based on the myotome phenotype in the MRF4 null mice (Patapoutian et al., 1995). Authors of that study presented a theory that recruitment of cells to the developing myotome occurs in three consecutive waves, the first initiated by Myf5, the second by MRF4, and the third by MyoD. While not disproved, it was not widely accepted, since the described MRF4 mutant allele contained the neomycin selection gene, which affected Myf5 gene expression in *cis* (Yoon et al., 1997). This theory may be reinforced in light of the present study, which has demonstrated MRF-independent activation of MRF4.

Further analysis of $Myf5^{\Delta loxP/\Delta loxP}$ mutants revealed that MRF4 expression is regulated in a different manner along the anterior-posterior axis. MRF4 is activated independently of Myf5 in myotomes of caudal, thoracic and posterior cervical somites. However, Myf5 seems required for MRF4 expression in the occipital and the anterior cervical somites. Neither the endogenous MRF4 nor the MRF4-III transgene were detected in these somites of Myf5-deficient mice at any developmental stage. A 2.8-kb region (+0.2/+3 kb) identified in the present study is necessary for driving expression of MRF4 in the same rostral somites, which are depleted of MRF4 transcripts in the Myf5 mutant. Therefore, it is reasonable to suggest that Myf5 may control MRF4 expression in the rostral somites through this region, either directly or indirectly.

Noteworthy, MyoD was expressed in these somites but was unable to rescue MRF4 expression. Thus, Myf5 and MyoD have functions that are not entirely redundant in the myotome of cervical somites. The myogenic cells from occipital and most rostral cervical somites contribute to all cranio-cervical muscles (Huang et al., 2000). As there is no detectable defect in the adult cervical muscles of Myf5 mutants, it seems that MyoD compensates for the lack of MRF4

in the rostral somites. Notably, MyoD/MRF4 double mutants have severe deficiencies in skeletal muscle cell differentiation (Rawls et al., 1998).

4.5. Myf5 is necessary for the formation of intercalated myotomes

The analysis of the expression pattern of endogenous MRF4 and MyoD in Myf5-/- embryos as well as MRF4 transgenes reveals defects in the myogenic program in intercalated myotomes. Similar defects were observed in the original Myf5 mutants (Braun et al., 1992; Tajbakhsh et al., 1997) based on expression of the MyoD transgene carrying the core enhancer and the myogenin transgene (Kablar et al., 1999; Kablar et al., 2003). Both, the MyoD and the myogenin transgenes were expressed exclusively in the dorsal and ventral portion of the myotome with a large gap in the central myotome.

The observed lack of myogenic factors in the intercalated myotome may be due to a specific Myf5 role in the differentiation program in this part of the myotome. As it was shown by Spörle (Spörle, 2001), several myotomal and dermomyotomal markers including Myf5 itself expose a selective expression profile in epaxial, hypaxial and intercalated domains in somite. Myf5 expression is activated initially in the epaxial and hypaxial dermomyotome lips, and then, during the subsequent elongation of dermomyotome, Myf5 is expressed continuously from dorsal to ventral limits of the myotomal layer. The strongest expression is in the central part of the epaxial myotome. Thus, different levels of Myf5 expression delimit the dorsalmost and central regions of the epaxial myotome from each other. From these observations, it was suggested by Spörle that this central region is composed of early epaxial myotomal cells, which arise dorsomedially and due to further growth of the myotome become intercalated between the more recently formed dorsal and the hypaxial myotome regions (Spörle, 2001). Several markers of differentiating myocytes, such as Connexin 40 or Frizzled 9, remain restricted to the intercalated myotome, whereas Wnt6, Wnt11 and Zinc finger of the cerebellum (Zic) 2 are expressed in the dorsalmost epaxial domain (Spörle, 2001). The intercalated region is marked by En1 expression in the abutting region of the dermomyotome. The existence of three distinct myotomal territories with respect to the expression pattern of myogenic

differentiation markers can possible indicate that myogenesis in the intercalated myotome follows a transcriptional program, which is different from that shared by epaxial and hypaxial somitic buds. This program may be impaired in the Myf5 deficient mice, since none of the myogenic markers is expressed in the central myotome (Fig. 29, C).

Alternatively, the deficiency of the intercalated myotome formation might be due to defects in cell migration (Fig. 29, B). The formation of new myocytes from the dorsal medial lip and the ventral lateral lip of the dermomyotome is apparently not affected in the Myf5 mutants, since the myotome normally grows dorsally and ventrally. However, the cell migration into the central myotome might be defective. Indeed, it was demonstrated previously for $Myf5^{nLacZ}$ mutant mice that progenitor cells are arrested in the dorsal and ventral domains and then migrate aberrantly (Tajbakhsh et al., 1996b). Recent studies revealed that $Myf5^{\Delta loxP/\Delta loxP}$ mutant fail to express FGF4 and FGF6 at early stages in the myotome (Brent et al., 2005). Given that FGFs are necessary for proper myoblast migration in vivo (Neuhaus et al., 2003), it may provide a molecular mechanism underlying the defect in migration of myogenic progenitors.

There is evidence that cells in the intercalated myotome may represent a distinct population of muscle progenitors (Teboul et al., 2002; Summerbell et al., 2000; Tajbakhsh & Spörle, 1998b). According to this hypothesis new myotomal cells arise progressively from the medial part of the dermomyotome (Tajbakhsh & Spörle, 1998b). Indeed, in situ hybridization analysis of young mouse somites revealed a third domain of Myf5 expressing cells that do not obviously belong to either the dorsal or ventral domains (Summerbell et al., 2000). This cell population originates in the central dermomyotome domain, specifically marked by En1 and Sim1. It should be mentioned, however, that expression of these markers is unaffected in the Myf5 mutant. GFP labeling of somitic borders in chicken demonstrated that the central region of myotome is composed of myocytes of rostral and caudal origin (Gros et al., 2004) It remains to be tested whether the defect in the intercalated myotome of Myf5 mutant is due to failure of myogenic progenitor to migrate into the central part of somites or because the tissue environment is unable to maintain the myogenic program there.

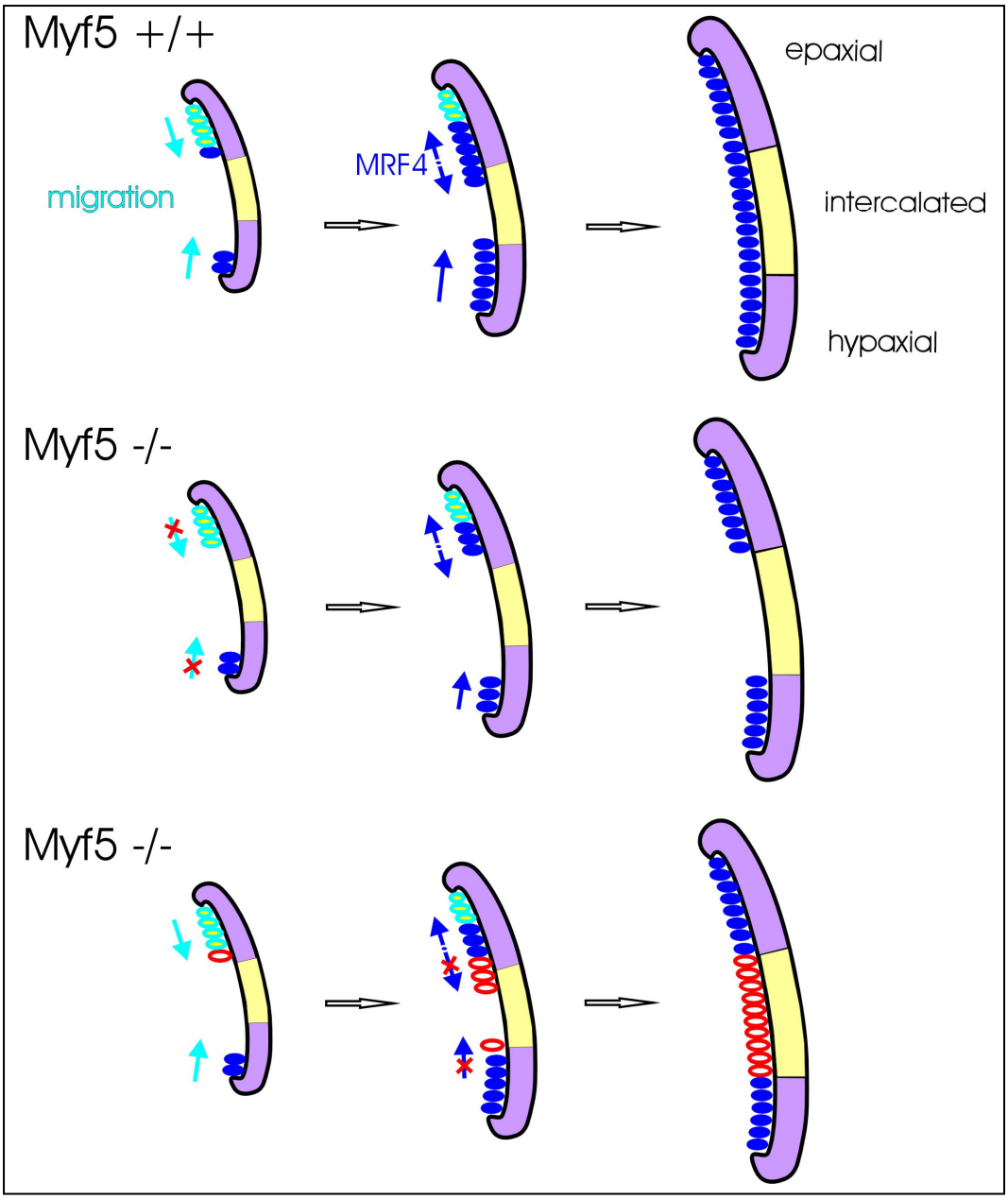

Figure 29. The defect in the central myotome formation of Myf5 mutants. Two hypothetical models are suggested to describe the mechanism underlying defect in the central myotome of Myf5 mutants. A: Normally myogenic precursors from the dorsal and ventral dermomyotome migrate towards the center forming intercalated myotome. MRF4 expression marks early myogenic progenitors. Its messenger appears first in the dorsal and ventral myotome and expression spreads both to the central myotome and to the most dorsal part of the epaxial domain, and to the center from the hypaxial domain, while cells migrate to the intercalated myotome. B: In Myf5 mutants the cell migration is affected. Progenitors are unable to migrate to the central myotome. As a result this domain fails to express MRF4. C: Progenitors migrate to the central myotome. However, the microenvironment is unable to support MRF4 expression that results in the myogenic program defect. Cyan arrows indicate directions of progenitors migration in the dermomyotome. Dark blue arrows show spreading of MRF4 expression. Cyan yellow-filled circles – cells that still not expressed MRF4, dark blue solid circles – MRF4 expressing cells. Red empty circles – precursors that failed to maintain MRF4 expression.

A subdivision of the myotome into three distinctly regulated compartments suggests individual roles of these regions for muscle development. In zebrafish the intercalated (adaxial) myotome is a source of slow muscle progenitors, which migrate radially. Specification of the adaxially developing myocytes depends on Hedgehog-mediated signals (Coutelle et al., 2001). In mouse Shh also functions in somites to establish and maintain the medio-lateral boundaries of epaxial and hypaxial gene expression (Borycki et al., 1999). However, the role of the intercalated myotome in the mouse myogenic program is presently not understood. Adult Myf5 mutant mice do not exhibit a significant muscle phenotype (T. Braun, personal communication). It seems, therefore, that defects in the intercalated myotome observed in the Myf5 mutant may be compensated by myogenic progenitors from the adjacent domains.

4.6. Conclusions

A novel 1-kb regulatory element that drives myotomal expression of the MRF4 gene was identified in this study using transgenic mouse technology. This provides now the opportunity to understand how expression of this myogenic factor is regulated. Computer based analysis predicts binding sites for transcription factors which mediate different signaling pathways. Functional assessment of these binding sites should clarify their role for MRF4 activation during myotome development.

Although this study demonstrates activity of the 1-kb regulatory element in context of transgenes, it does not show whether this element is essential for correct MRF4 expression in vivo. Loss-of-function mutants would be critical to directly evaluate the functional importance of this element during normal development.

MRF4 expression is regulated differently in the myotome and in fetal muscles. This study demonstrates that, additionally to previously described regulatory regions, sequences located downstream of the MRF4 gene have substantial influence on fetal MRF4 expression. Detailed mapping and characterization of these sequences will be required to provide further insights into MRF4 regulation in fetal and adult muscles.

MRF4 is located in close proximity to the Myf5 gene in the locus. However, the limb specific enhancer activates only the Myf5 gene in the limb. The present study demonstrates that the Myf5 specific limb enhancer could activate the MRF4 promoter when juxtaposed. Future investigations should elucidate the mechanism by which the enhancers discriminate between different genes in the locus.

The analysis of the Myf5 mutant revealed the role of Myf5 in the central myotome formation. Further studies should be performed to establish cellular mechanism underlying the defect in the intercalated myotome. It would be also interesting to trace the cell fate of cells from intercalated myotome in the adult mouse.

This study demonstrated that activation of MRF4 in the ventral and dorsal domains of myotome is independent of Myf5 and MyoD. Therefore, the role of MRF4 in myogenesis and its place in the hierarchy of the myogenic regulatory factors should be reevaluated. It is important to determine place of MRF4 in the network of factors inducing the myogenic program. To this end, a careful examination of the MRF4 expression pattern should be performed in the mice with mutations compromising corresponding signaling pathways.

Table of Figures

Abbreviations

BAC	bacteria artificial chromosome
bHLH	basic Helix-Loop-Helix
bp	base pair
cDNA	complementary DNA
Cre	center of recombination (of phage P1)
DIG	digoxygenin
dNTP	deoxyribonucleoside triphosphate
E	Embryonic stage = day post coitus
EDTA	ethylenediamintetraacetate
FGF	fibroblast growth factor
Fig	Figure
g	gram
hCG	Human chorionic gonadotropin
HLH	Helix-Loop-Helix
IU	International units
kb	kilobase pair
l	liter
LacZ	β-galactosidase
LCR	locus control region
loxP	locus of crossing over (of phage P1)
M	mole
MABT	maleic acid buffer + tween
MEF	myocyte enhancer binding factor
mg	milligram
µg	microgram
min	minute
ml	milliliter
µl	microliter
mM	millimole
MRF	Myogenic Regulatory Factor
mRNA	messenger RNA
nLacZ	nuclear β-galactosidase
NTMT	Na Tris Magnesium Tween buffer
PBS	phosphate buffered saline
PCR	Polymerase Chain Reaction
PK	Proteinase K
PMS	Pregnant mare serum gonadotropin
RT	room temperature
RT-PCR	reverse transcriptase PCR
SDS	sodium-dodecyl-sulfate
sec	second
ss	somitic stage
SSC	Saline Sodium Citrate buffer
ssDNA	single-strain DNA
YAC	yeast artificial chromosome

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1991-1992	TEL-AVIV UNIVERSITY, Israel Studies towards B.Sc. at the Faculty of Life Sciences
1992-1995	Master of Science at the TEL-AVIV UNIVERSITY, Israel Sackler School of Medicine. Major: Cell Biology and Histology. Thesis: "Potential of fibroblastoid cell lines from the developing bone implants to secrete hemopoietic growth factors and influence of different biological compounds on the function of these cells."
2000-2005	Studies towards PhD degree at the TECHNICAL UNIVERSITY of Braunschweig, Germany Department of Cell and Molecular Biology, Institute of Biochemistry and Biotechnology Supervisor: Prof. Dr. H.-H. Arnold. Thesis: "Transcriptional regulation of MRF4 gene expression during embryonic mouse development."

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